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COLD SPRING HARBOR
SYMPOSIA ON
QUANTITATIVE BIOLOGY

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QUANTITATIVE BIOLOGY

VOLUME VII
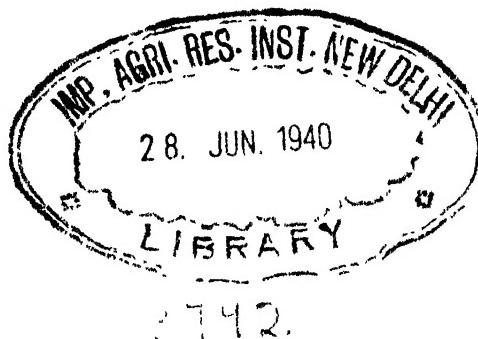
COLD SPRING HARBOR, L. I., N. Y.
THE BIOLOGICAL LABORATORY

1939

COLD SPRING HARBOR SYMPOSIA
ON QUANTITATIVE BIOLOGY

Each summer the Biological Laboratory at Cold Spring Harbor invites a group of mathematicians, physicists, chemists, and biologists to take part in a Symposium upon some selected aspect of quantitative biology. Each Symposium lasts for five weeks, and the participants live at the Laboratory for a part or all of the time. The meetings are conducted with a minimum of formality, and no limit is placed either on the length of papers or on the discussions following them; indeed, much of the discussion inevitably takes place between individuals after the meetings at which the papers are read. The discussions are then revised by those taking part in them, and so the resulting volumes give a true picture of the opinion of the group at the time the volumes are prepared. The number of people invited to give papers is purposely kept small, and every effort is made to prevent the meetings from becoming mere "lectures" by discouraging any but experts from taking part in them. The immediate value of these Symposia is obviously greatest to those taking part, but at the same time the published volumes make the papers and discussions available to scientists at large with the least possible delay, and at less than the cost of publication alone.

The subjects for the Symposia are selected on the basis of their being subjects in which rapid advance has recently taken place along quantitative lines. Thus the Symposium of 1933 dealt with surface phenomena, that of 1934 with aspects of growth, that of 1935 with photochemical processes, that of 1936 with excitation phenomena, that of 1937 with internal secretions, that of 1938 with protein chemistry, and the present one deals with biological oxidations. It ought to be pointed out that in preparing the program and in selecting the material for publication, comparatively little attention is given to the subject being as comprehensively covered as it would be in a monograph or text book. Some of the papers may be of the nature of reviews, but the majority are concerned with a presentation of specialized and even controversial aspects of the subject, and assume that the background is familiar to the reader. It is true that a probable result of this is that the volumes will be out of date within a comparatively few years, but to research workers such a disadvantage is outweighed by each volume presenting the state of the subject as it exists at the moment, and presenting not only what is known, but what is still speculative or undetermined.

ERIC PONDER

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CONTENTS

SOME ELEMENTARY ASPECTS OF THE POTENTIOMETRIC STUDY OF OXIDA-TION-REDUCTION EQUILIBRIA

W. Mansfield Clark

When I see the faces of so many who are familiar with the subject to be reviewed I feel embarrassed. So many reviews already have been published. But Dr. Ponder requested this one as an introduction to the Symposium and I accept the task feeling that in the elementary principles of the subject reside implications that are worthy of reiteration and that have not yet been realized fully. Someone is said to have characterized a former review of mine as "the same old equations". This characterization was literally correct, and yet the flat note of the utterance is impatience with a subject that can proceed no step beyond what its quantitative data will yield when they are put in order by its theoretical formulations.

Let me confine an historical introduction to a brief reminiscence of a remarkable simultaneity of resurgent interest in potentiometric studies. Quite independently and at essentially the same time four or more groups of investigators entered the field. At Columbia University, Granger (1920) of Nelson's laboratory and, in Copenhagen, Biilmann (1920) extended a potentiometric study of the benzoquinone system that had been initiated but inadequately treated by Haber and Reuss (1904). Meanwhile Conant had planned the investigation of quinones on which he and his students first reported in 1921 (see Conant, Kahn, Fieser and Kurtz, 1922) and during a trip to Europe he found another laboratory engaged in a similar study that appears never to have been published. My own part in this resurgence began when, in 1919, my friend Louis Gillespie told me his observations of the changes of electromotive force in a cell one half-cell of which was composed of a mercury electrode and a culture of bacteria. When I finally saw the implications I insisted on his publication of the observations with a friendly threat to steal the field if he did not publish. With Gillespie's encouragement I made literally hundreds of measurements with various cultures but soon reached the conclusion that a better theoretical understanding of the phenomena was more important than a multitude of measurements. Working as I was under the inspiring leadership of L. A. Rogers, a bacteriologist, it was only natural that I should turn to the study of reagents that had been used to detect reduction in bacterial cultures. Thence came the initial study of methylene blue and indigo carmine (Clark, 1920).

Much later Cannan, Cohen and Clark (1926) found courage to extend and to publish a little of the work on cultures. At that time there was little evidence of those specific substances to which

might now be attributed the electromotive forces that Gillespie (1920) and we had observed.

What a contrast between the states of knowledge then and now! In my opinion the most important biochemical work that has been done in the meantime has been the isolation and identification of several components of the living cell that act as catalysts and several that act as intermediates in oxidative metabolism. This has given substantiality to a subject of which it was formerly said that none in the whole field of biochemistry was more burdened with speculation based on inadequate experimental evidence.

What I shall review may be relatively of secondary importance, and yet within limitations it can serve as a corrective to what has been and in some degree remains an overemphasis of the desire to explain mechanisms.

Let us first consider the terms *oxidation* and *reduction*. After Lavoisier had used Priestley's discovery of oxygen to reconstruct the theory of combustion it became natural to speak of the formation of calces from metals as a process of oxidation. The reverse process, such as lowering the degree of oxidation from ferric oxide to that of ferrous oxide and finally to metallic iron, now naturally might be called a process of reduction—reduction of the degree of oxidation. But the ancient metallurgists used the term in referring to the reformation of the metal from the calx. As indicated in Fig. 1, ferrous chloride on the one hand and ferric chloride on the other can be obtained by dissolving the respective oxides of iron in hydrochloric acid and separating the products in the solid state. By referring each of these chlorides to the respective oxide, ferric chloride may be regarded as a product of the oxidation of ferrous chloride. The conversion can be effected directly by chlorine and, according to this system of reference, chlorine may be considered an oxidizing agent. Ferrous and ferric chlorides are here mentioned as substances known in the solid state. Emphasis may be placed upon the last statement because the failure to differentiate between substances as they occur in the solid state and in solution still causes confusion. For example, biochemists habitually use the concept of the complete dissociation of sodium bicarbonate and yet write $NaHCO_3$ in their equations describing equilibrium states in blood. The retention of several similar habits that have been fixed by the historical order of progress rather than by rational readjustments thereto leaves the elementary student confused.

In the case of the ferric and ferrous chlorides now under consideration as prototypes, the re-

Oxidation →
← Reduction

Addition of Oxygen	$2FeO + \frac{1}{2}O_2 \rightleftharpoons Fe_2O_3$
Solution in HCl and isolation of chlorides	↓ ↓
Addition of Chlorine	$2FeCl_2 + Cl_2 \rightleftharpoons 2FeCl_3$
Ionization (neglect Cl^-)	↓ ↓
Loss of Electron	$2Fe^{++} \rightleftharpoons 2\epsilon + 2Fe^{+++}$

| Loss of Hydrogen or | Indigo white $\rightleftharpoons$ indigo $+ 2H$ |
| Loss of $2H^+ + 2$ Electrons | Indigo white $\rightleftharpoons$ indigo $+ 2H^+ + 2\epsilon$ |
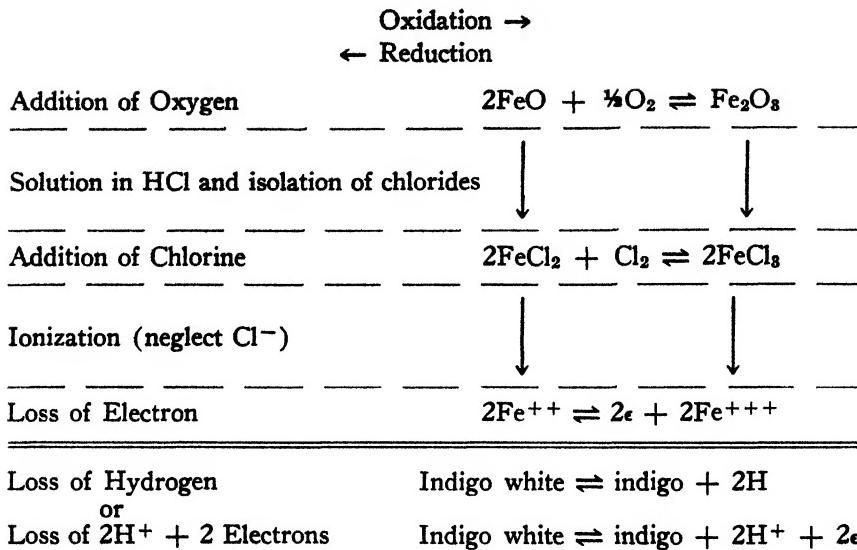
FIGURE 1

presentation given by Arrhenius' theory was that they are partially dissociated to ions in aqueous solution. Of a later date, we have to consider more carefully both the extent of this dissociation and the coordination of the metal ions with water. If attention be focused upon the simplest representation of the metal ions it is seen that they differ by one electric charge, now represented as an electron. By extending the system of reference we may consider the loss of an electron to be an oxidation and the gain a reduction.

Also we have to consider changes of organic compounds. Nothing was plainer to the older chemists than the change to blue as the cloth came from the vat of the indigo dyer. After the discovery of oxygen the conversion of indigo white to indigo blue was plainly an oxidation, and after organic analyses had established that the mild oxidation of many organic compounds changes their content of hydrogen (as examined in the *solid* state!) the removal of hydrogen fell into the category of the oxidative process. In the same category can be placed processes typified by the Cannizzaro reaction, for, if we are not too narrow in restricting the significance of this reaction, we can place in this class those changes within a molecule whereby one part is oxidized and the other reduced.

All this is far less than a scheme of mechanisms and far more than a merely arbitrary system of classification. The fact that a substance can be changed to the same product by oxygen, chlorine, or metallic ion signifies a community of properties among these agents. This is expressed by chemists in their generalization of the term oxidation. To say that a particular process operates exclusively is merely to express ignorance of the fact that during the evolution of the generalized view good uses have been made of the convenience of the simplest description appropriate to a particular case. To make use of such a convenience is not to describe a mechanism. And yet something more now can be done by way of judging probabilities from data of the sort I shall present and from other sorts of evidence. I shall illustrate this later. Let us now turn to the main subject.

For present purposes we may describe the total energy change, ΔU, in a system by the following equation

$$\Delta U = T\Delta S + P\Delta V + \mu_1\Delta N_1 + \mu_2\Delta N_2 + \mu_3\Delta N_3 + \text{etc.} \quad (1)$$

Δ: read increase of—
T: absolute temperature
S: entropy
P: pressure
V: volume

μ_1, μ_2, μ_3, etc. are the chemical potentials of the individual components whose mole fractions are N_1, N_2, N_3, etc.

If $P\Delta V$ be moved to the left of the equation we have there $\Delta U + P\Delta V$, which sum is called ΔH. Since $T\Delta S$ is left on the right, ΔH, which can be determined by differences in heats of combustion at constant pressure, is not indicative of the change in the sum of the $\mu\Delta N$ terms. To obtain the latter both $P\Delta V$ and $T\Delta S$ have to be moved to the left. Then the algebraic sum $\Delta U - T\Delta S + P\Delta V$ is indicative of what may be called the changes in the chemical energies of the system's chemical components. This sum is called the change of free energy, $\Delta F = \Delta U - T\Delta S + P\Delta V$.

For reasons discussed in all texts of thermody-

namics, ΔF, under proper limitations, is what determines the direction of a spontaneous chemical change when a path for that change is open. Hitherto those who have attempted to apply thermodynamics to biochemical systems have been restricted largely to the use of values for ΔH because the more useful values of ΔF were unknown. In some instances the estimated differences are small; in others not. For the transformation of a mole of succinate to a mole of fumarate ΔH is 29,800 calories and ΔF is 20,400 calories. For the transformation of a mole of lactate to a mole of pyruvate ΔH is 21,600 calories and ΔF 11,400 calories. In these cases the differences are considerable.

To obtain a value of ΔF by the thermal method involves the difficult measurement of ΔS as well as of the more easily determined value of ΔH. On the other hand the electric cell sometimes provides a device whereby a free energy change can be directed into an electrical channel and measured with high precision.

We may conceive of the cell shown in Fig. 2
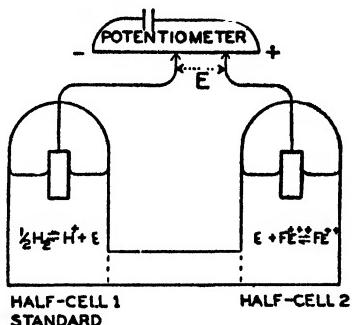
FIGURE 2

as follows. It contains two oxidation-reduction systems kept from direct interaction by spatial separation. The reductant of each of these systems is inherently capable of releasing one or more electrons and the oxidant of receiving electrons. The differential effect is a characteristic electron escaping tendency of the system which is composed of oxidant and corresponding reductant. This escaping tendency is expressed, for the purposes of treatment in terms of energetics, as an electron chemical potential. The device of the electric cell provides in part of its circuit a metallic path that can filter electrons from the other components and permit them to flow from the system of greater electron chemical potential to that of lower electron chemical potential, provided there be proper compensation for the electrostatic charge that would result from this were it the only change. Such compensation is accomplished by the migration of ions in the liquid junction. For purposes of first approximation we shall assume

that the liquid junction potential resulting from unequal migration velocities of the ions is managed in such a way as to be negligible.

For a brief outline of the derivation of equations with the aid of this conception of the cell I refer you to my Harvey lecture (Clark, 1933). All that need be said here is that, if we restrict the objective by avoiding considerations that bear upon the mechanism of electrode processes, we can relate the potential difference very directly to the free energy change in the action of one system on the other. Here it may be emphasized that use is being made of a very special device. When the configuration of this device is removed the special path for the interaction of the one system on the other is removed but the inherent potentiality of interaction is not. This is expressed by the free energy change. Any other path leading from the initial to the final products should involve the same energy change. Since a critical examination of the thermodynamic equations, of limitations in their application to cells with liquid junction and of experimental technique would occupy too much time and is available elsewhere, I shall pass directly to "classical" equations which, if cautiously used, suffice to describe the data with which a "map" of our subject is "roughed out".

The equation applied to such a cell as that depicted in Fig. 2 is

$$(2)$$
$$E_h = E_o + \frac{RT}{F} ln \frac{\sqrt{H_2\ \text{pressure}}}{[H^+]} + \frac{RT}{F} ln \frac{[Fe^{+++}]}{[Fe^{++}]}$$

It is agreed that the oxidation-reduction system of which molecular hydrogen is the reductant and the hydrogen ion the oxidant shall be a standard of reference when the hydrogen pressure is one atmosphere and the hydrogen ion concentration (strictly, the hydrogen ion activity) is unity. When these specifications are observed, the second term on the right side of equation (2) is zero and

$$E_h = E_o + \frac{RT}{F} ln \frac{[Fe^{+++}]}{[Fe^{++}]} \qquad (3)$$

$$E_h = E_o, \text{ when } \frac{[Fe^{+++}]}{[Fe^{++}]} = 1.$$

What is presented first is a series of measurements with cells in which each of several oxidation-reduction systems in turn replaces the iron system shown in Fig. 2 and the hydrogen system is kept at the state of the standard of reference. Then, in general,

$$E_h = E_o + \frac{RT}{nF} ln \frac{[\text{Oxidant}]}{[\text{Reductant}]} \qquad (4)$$

For each case E_o is unique and is equal to the measured value of E_h when the ratio of concentrations of oxidant to reductant is unity. A complication will appear presently. As the degree of reduction (or oxidation) of the system is changed, E_h follows a sigmoid curve specified by equation (4) and illustrated by Fig. 3. In this figure experimental points are shown by centers of circles and theoretical curves by lines. Incidentally I suggest that in dealing with a subject that has become so precise we avoid smoothing empirical

FIGURE 3

FIGURE 4

curves and give only theoretical curves and experimental data as points. This will avoid confusion.

In Fig. 4 are two examples of inorganic systems in one of which the "valence change" is 2 and in the other 1. By using other evidence to exclude dimerization we have proof in the distinctive slope of the thallium curve of the two equivalents per gram atom. The conclusion from the slope of the curve for the iron system is that one equivalent is concerned. Since such slopes are to be used by Michaelis in his discussion of step-wise oxidation-reduction of some organic compounds I need not discuss further this use of the slope.

Before we continue it may be well to say that while we might express our free energy data as volt-faradays (EF) or, if preferred, as calories, there is good reason to retain the directly measured quantity, potential difference. Electrical potential, gravitational potential, pressure and temperature (which happen historically not to have been given the formal dimensions of a potential) have the *properties* of a potential. As heat will flow from a body of higher temperature to one of lower temperature, so electricity will flow from a body of higher electrical potential to one of lower when a path is provided. Likewise electrons will flow in our special device from the system of higher electron chemical potential to the system of lower electron chemical potential. It is this factor, then, that determines the direction of action in the electric cell or when two active oxidation-reduction systems are directly mixed. The extent of action is easily calculated with the aid of additional knowledge of the quantities of the components concerned in a particular case.

In Fig. 5 are assembled the curves characterizing various oxidation-reduction "indicators". Within the category of reactions to which they apply these indicators may be used in several manners analogous to the modes of use of acid-base indicators. Cohen (1933), at a former Symposium held here, reviewed their uses in exploring the interior of cells. I first used them in checking what electrode measurements revealed of cell suspensions. Rational selections can be made the bases of titrimetric analyses such as the estimation of ascorbic acid. These systems have served as "mediators". Being "electromotively active", which is only a way of expressing the fact that an electrode responds to the state of such a system, they can stabilize an electrode potential. Then if they happen also to react with a system that is not "electromotively active", the connotation of the term *mediator* is plain. If such a system enter into equilibrium with another, the colorimetric estimation of the equilibrium constant and the possession of a quantitative value for the characteristic potential of the dye system permits

ALL SYSTEMS AT PH = 7.0

|	
0 273	PHENOL-M-SULFONATE-INDO-2,6-DIBROMOPHENOL
0 254	M-CHLOROPHENOL-INDO 2,6-DICHLOROPHENOL
0 248	M-BROMOPHENOL INDOPHENOL
0 233	O-CHLOROPHENOL-INDOPHENOL
0 224	BINDSCHEDLER'S GREEN
0 217	2,6-DICHLOROPHENOL-INDOPHENOL
0 181	2,6-DICHLOROPHENOL-INDO-O-CRESOL
0 172	1-NAPHTHOL-2-SULFONATE-INDOPHENOL
0 119	INDO-2,6 DICHLOROPHENOL
0 115	TOLUYLENE BLUE
0 047	CRESYL BLUE
0 021	GALLOCYANINE
0 011	METHYLENE BLUE
-0 036	CIBA SCARLET SULFONATE
-0 046	INDIGO TETRASULFONATE
-0 060	METHYL CAPRI BLUE
-0 081	INDIGO TRISULFONATE
-0 125	INDIGO DISULFONATE
-0 142	GALLOPHENINE
-0 173	BRILLIANT ALIZARINE BLUE
-0 252	PHENOSAFRANINE
-0 273	TETRAMETHYL PHENOSAFRANINE
-0 289	SAFRANINE T
-0 299	INDULINE SCARLET
-0 325	NEUTRAL RED
-0 385	ROSINDONE SULFONATE NO 6
-0 4207	HYDROGEN, 1 ATMOSPHERE
	REGION OF HYDROGEN OVER-VOLTS

FIGURE 5

the calculation of the free energy change (or potential factor thereof) in the conversion of a mole of the oxidant of the second system to a mole of its reductant. They have served in a true sense as catalysts of graded and well defined potential energies.

Let us now go back for a moment to a proposition on which I promised to comment. I said that historically good uses had been made of the convenience of the simplest description of a mechanism appropriate to each particular case. The question is: can we use data of the sort just described to judge the extent to which particular processes are operating? Imagine a solution containing as possible components the oxidants and reductants of several oxidation-reduction systems. If these systems were in equilibrium and if the potential of the solution were known it should be possible to calculate the ratio of oxidant to reductant for each system. Such a calculation might show the ratio for one or another system to be so high or so low that, for all practical purposes, concentration of a certain oxidant or reductant is negligible. By extending this proposi-

tion to include any of the special material components exclusively in terms of which special theories of biological oxidation-reduction have been concocted, one sees at once the weakness of these theories.

In Fig. 3 and 5 the pH values of the solutions have been carefully specified for a reason that now must be made plain.

If the methylene blue system be placed in a series of buffers and, in each instance separately, the change of potential with change in degree of oxidation be measured, the series of sigmoid curves shown in Fig. 6 B will be obtained. The result of plotting pH against the potentials at center points of these curves is shown in A. To obtain a figure the coordinates of which are the three quantities, per cent oxidation, pH and E_h, place the plane of B perpendicular to the plane of A and move the centers of the curves B to their positions on A. Figure D results.

To describe the pH effect in chemical terms consider the potential:pH curve of the benzoquinone system at 50 p.c. reduction (Fig. 7). In the electric cell we may imagine electrons to flow through the metallic part of the circuit from the reference, hydrogen system of higher electron escaping tendency to the quinone-hydroquinone system. Were the electrons alone to be added to quinone this would convert quinone to the anion of hydroquinone. At high hydrogen ion concentrations, however, the anion of hydroquinone can pick up protons easily from components of the solution, and become hydroquinone. As pH increases, this ease of pick-up declines. In other words, more energy is used in extracting protons
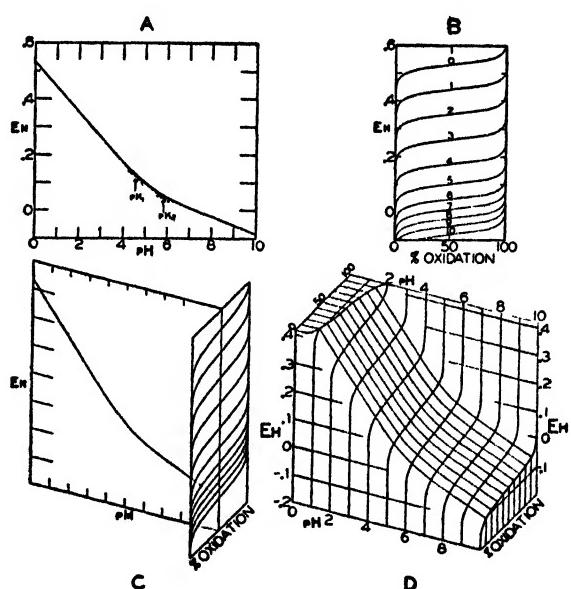
FIGURE 6
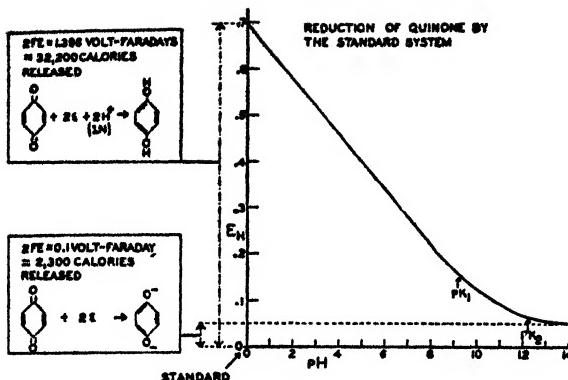
FIGURE 7

FIGURE 8

from the solution. This makes itself felt in a decline of the energy yielded by the operation of the cell, or in the over-all potential difference. Expressed without resort to this mechanistic view and by an equation that may be derived with the aid of any one of a half-dozen postulated mechanisms, it may be said that what is being measured in this instance is the "free energy of dilution" of hydrogen ions or the equivalent thereof which is the energy of extraction of protons without specification of the degree to which they are free. At very high values of pH the solution will spontaneonsly strip protons from the phenolic groups of hydroquinone and no further change of potential will then occur. By proper use of these characteristics the energy of ionization can be measured. In other terms, intersections of projections of the branches of the curve occur at pH values equal to the pK_a values. Since each system is composed of constituents having uniquely characteristic groups to which pertain special ionization constants, there is great variety among the E'_0:pH curves (see Fig. 8).

It is now evident that the potential measurements automatically integrate the free energies of oxidation-reduction, of hydrogen ion dilution, and of acid ionization, and partially to resolve these requires control of the several factors.

That the integration obtains in the absence of the configuration of the electric cell and in accordance with predictions based upon measurements made with the electric cell can be shown by any one of several simple experiments. As illustrated in Fig. 8 the E'_0:pH curve for a ferro-ferricyanide mixture crosses that of the 2,6-dibromophenolindophenol system. It is possible to prepare a solution, containing the components of these two systems, such that the phenomenon predictable with the aid of Fig. 9 will occur. On addition of acid the dye is reduced; on addition of alkali the oxidation-reduction process is reversed.

Perhaps in accord with the adage "familiarity breeds contempt", or with the fact that the formula-
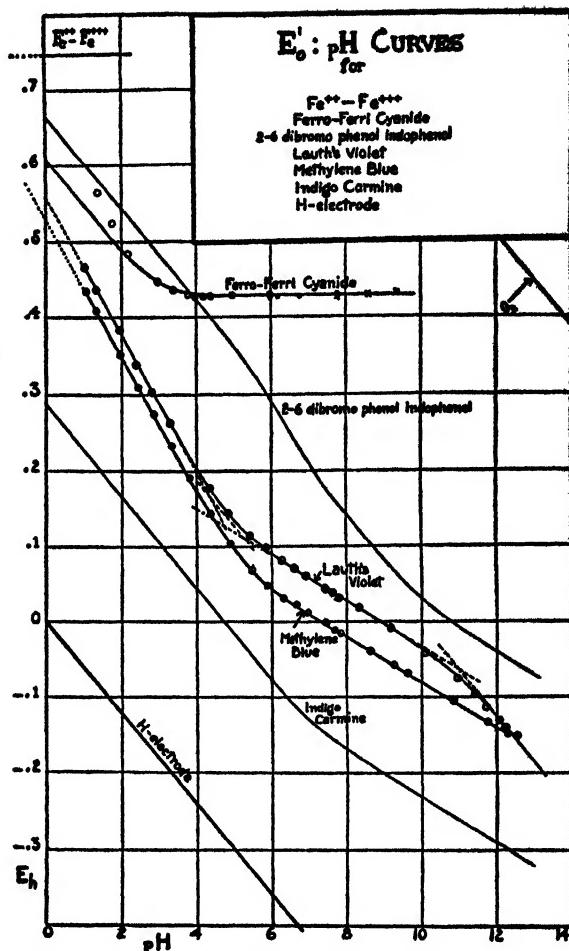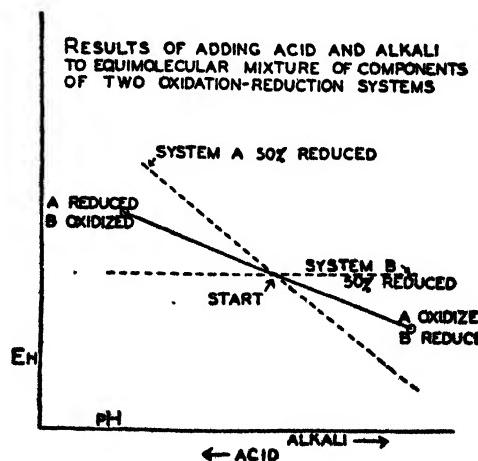
FIGURE 9

tion is so precise as to do away with the attractive features of pure speculation and to force the hard work of accumulating the quantitative data demanded, this principle has not received the attention it deserves as a prototype of the coupling of processes of different types. In biochemistry we have pressing reasons for looking into the coupling of processes of different sorts and shall need information on what couplings are thermodynamically possible and actually true. Speculation thereon has its place in the mind of him who plans bold adventures; ultimately speculation will have to give place to quantitative data of the character, if not the kind, here illustrated.

It has long been recognized, particularly in the field of inorganic chemistry, that the possession of a table of so-called normal potentials enables one to predict the direction of interaction of any two oxidation-reduction systems and that knowledge of the absolute amounts of reacting components enables one to complete the calculation and give the extent of a given reaction. Latimer (1938), in the preface to his recent book, *Oxidation Potentials,* wherein are compiled all available data for inorganic systems, remarks "... a table of the energies of the elements in their various oxidation states may be used by one with a very elementary knowledge of thermodynamics to answer many of the qualitative questions involved in the interpretation of inorganic chemistry."

Of primary interest are such data for systems occurring in the cell. Many such systems have now been measured, notably the various quinone systems occurring in plants, bacteria or in our own bodies, the metalloporphyrins and metalloproteins, flavins, etc. These systems may be said to be electromotively active in the sense that without the intervention of any catalyst an electrode responds quickly to a change in the ratio of oxidant to reductant. Other systems, such as that of which ascorbic acid is the reductant, affect the electrode less readily and are sometimes called "sluggish". Many such systems have long been known in the inorganic field and occasionally have been studied with the aid of a mediator. Ball (1937) successfully employed electromotively active mediators in determining the characteristic potentials of the ascorbic acid system, and Taylor is now using the same principle in Hasting's laboratory to obtain more reliable data for the hemoglobin-methemoglobin system.

Other systems, of which the standard hydrogen-hydrogen ion system is typical, require a catalyst, in this case platinum, or palladium black. In other cases certain catalysts found in living cells work well.

A remarkable extension of the uses of the electric cell has been made by use of biological catalysts and in the following manner. If one

exposes a platinum electrode to a solution containing succinate and fumarate no stable potential will obtain. The addition of a mediator merely results in the response of the electrode to the state of the mediator and this is unaffected by the succinate-fumarate system. But if there is added a specific catalyst called "succinic dehydrogenase", obtained from certain bacteria or muscle, it was shown by Lehmann (1930) that the electrode responds to those changes in the ratio of succinate to fumarate and in pH that are predicted from the nature of the expected reaction. Substrate system, catalyst and mediator are involved.

This first case of its kind has been checked in various ways. Quastel and Whetham (1924) and Thunberg (1925) used the biological catalyst to accelerate the attainment of equilibrium between the succinate system and the methylene blue system. By use of the equilibrium constant and the potential data characterizing the methylene blue system it was possible to calculate the free energy change in the conversion of succinate to fumarate. Lehmann's direct potentiometric measurements were confirmed by Borsook and Schott (1931) who also applied the third law to the estimation of entropy changes and, with the additional help of heats of combustion, calculated the free energy change by these thermal data. Also use of the temperature coefficient of the electromotive forces and of the Gibbs-Helmholtz equation permits the calculation of ΔH. More direct estimation of ΔH by the thermal route comes by way of differences in heats of combustion at constant pressure. As indicated in Table I the agreements are excellent —almost too good.

It should be mentioned that in some of these calculations are involved several steps, account being taken of energies of solution, ionization, etc. Some of this complication can be avoided and the energy change under a particular set of conditions can be determined more directly when the device of the electric cell is applicable.

Without now stopping to accredit those who have extended this method or to bring the assembly of data up to date I show in Fig. 10 at the left a few of the potential curves characterizing some of the intermediates that occur in one or another set of the oxidative processes of metabolism.

There should be no misunderstanding of this retention of the dimension, electrode potential. Its retention does not mean that we attribute to the living cell anything comparable to the phenomenon of the electric cell. It does mean that there has been measured what is sometimes called the "intensity factor" of the free energy change relative to the standard system. As height of land relative to the sea is the standard mode of expression, so this system of referring potentials to the hydrogen

TABLE I

Oxidation of Succinate to Fumarate

Succinate ion⁻(1M) → Fumarate ion⁻(1M) + 2H⁺(1M) + 2ε

$$-\triangle F = -20,140 \text{ calories}$$
$$= -84,270 \text{ volt coulombs}$$
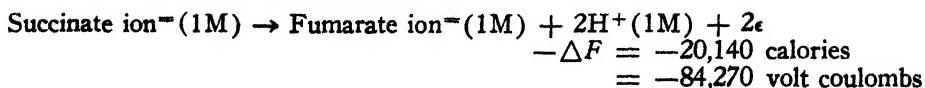
Heat Change (Difference in heats of combustion)
Succinate ion⁻(1M)→ Fumarate ion⁻(1M) ; $-\triangle H = -29,800$ calories
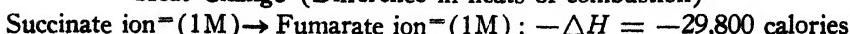
Data reduced to 25°C. basis by Borsook and Schott

Investigators	Method	$-\triangle F$	$-\triangle H$
		calories	calories
Quastel and Whetham (1924)	Equilibration with methylene blue*	—20,180	
Thunberg (1925-1928)	Equilibration with methylene blue*	—20,100	
Lehmann (1929-1930)	Potentiometric with mediators	—20,180	—29,850
Borsook and Schott (1931)	Potentiometric with mediators	—20,140	
Borsook and Schott (1931)	Thermal	—20,460	—29,800

* Data for methylene blue by Clark, Cohen and Gibbs (1925).

system and in terms of potential is a standard mode of expression. As differences in "sea level" can be used to predict the flow of streams of water, so differences of electrode potential can be used to predict the direction of energy flow.

We are now dealing with the chief use to which the measurements will be put as the subject de-

FIGURE 10

velops, since we are at the heart of the subject. In a later address at this Symposium Ball will make certain modest uses of the still meager data that are available, so I shall not pause to illustrate.

There are certain cautions that flow from elementary principles. Each of the sigmoid curves relating potential to degree of oxidation-reduction is asymptotic to the potential axis. It follows that, if a component of a system is withdrawn from the field in any physical or chemical process whatsoever, the state of that system may be defined by one or the other asymptotic part of its curve. Then, in comparing the curves characteristic of two systems, false predictions may be made if only the relative potentials at the flat portions of the curves are compared. Ball and Chen (1933) have illustrated this with the epinephrine system, the oxidant of which in neutral solution has an amazing instability and disappears quickly. Because of this, epinephrine can be extensively oxidized by a system the "normal" potential of which is distinctly below the normal potential of the epinephrine system. Similar considerations have been given to the oxidation of hemoglobin to methemoglobin, in the presence of cyanide, by systems of normal potentials lower than that of hemoglobin-methemoglobin system.

Since it is to be expected that a substance formed in one part of a metabolic process promptly may be transformed in another part, it is obvious that very severe limitations to the too simple application of the free energy data will have to be considered. There is danger that
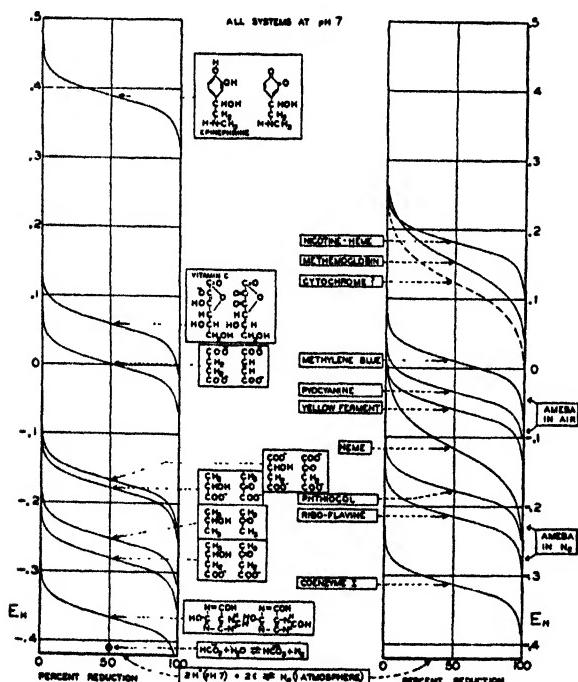
errors of interpretation may be due, not to errors of the free energy data, but to false uses.

Also the available data are for systems in homogeneous solutions. The living cell is not such a solution. Undoubtedly Korr will discuss some consequences at this Symposium.

The user of the electric cell is continually confronted by the limitations to its applicability. Knowing little of any fundamental importance as to why this is true, we may say with Gibbs that some systems exhibit "passive resistance". While it is true that the use of biocatalysts has partly broken through this natural barrier set against the wide use of the electric cell, there remain some aspects of this subject that deserve mention.

In the first place there may be, and probably is, no necessary connection between resistance to the display of changes on which the action of the electric cell depends and resistance to direct interaction between two systems which are in direct contact. In other words, factors on which electromotive activity depends may be special and electromotive activity may not be a universal criterion of chemical activity. Therefore we shall be wise to cite the passive resistance to activity in the electric cell merely as an example of a phenomenon of wider bearing.

In the field of the living cell passive resistances keep the components from that complete interaction which would lead to the dead level of a true equilibrium state. Thus are preserved reserves of energy for future use. Perhaps when we shall have learned enough of molecular and atomic forces to give concreteness and substantiality to this aspect, the subject of passive resistance may became as important to biochemistry as catalysis now is.

In the meantime the following minor points may be noted. The reserve energy mentioned above is the potential energy of chemical systems. Irrespective of the modes of action we need to know the potential energies of the individual systems referred to some standard, for these either wholly determine the direction of energy release or else limit the kinetic and other factors. This states in terms of potential energy the main theme of the present subject.

The fact of passive resistance gives to the study of oxidation-reduction in the biological field an aspect entirely different from that of the study of proton transfers. The latter are so rapid in systems of acids, bases and salts that they can be treated almost exclusively in terms of equilibrium states. The realization that this sort of treatment cannot be applied exclusively to oxidation-reduction processes turns the attention of biochemists toward the elucidation of paths, mechanisms and catalysts.

Thus we biochemists are forced to consider all of these subjects. While I fully realize the danger of an approach to affairs involving kinetics by way of a subject that is safe only when treated in terms of energetics, I think we cannot be less bold than students of inorganic chemistry who have in some instances coupled the energetics of electric cells with the kinetics and mechanisms of reactions.

Therefore, let me comment conservatively upon some modest uses of our subject in these other fields.

One use is typified by Ball and Chen's (1933) measurement of the rate of change of oxidized epinephrine. We may omit description of the special apparatus used and emphasize the following. The temporary equilibrium state of the system is reflected so quickly in the electrode potential that changes in the latter can be used to follow the disappearance of the oxidant. In other words the equilibrium data furnished a base line for a study in kinetics.

It is an experimental fact that the addition of a third system to two that interact slowly will sometimes accelerate action. I have previously (1937) cited several instances, notably those given by Barron, Green and others, and have attempted to express one possible view of this phenomenon in established terms that do away with the necessity for any additional nomenclature.

To be concrete let us consider the lactate-pyruvate and the succinate-fumarate systems, each "activated" by their respective enzymes, yet under circumstances such that there is little action between the two. Now add a mediator that can act with the components of each system. While the system of higher potential is oxidizing some molecules of the mediator, the system of lower potential is reducing those molecules of mediator that are available as oxidants. The final trend must be toward a common equilibrium potential. If, initially, unequal rates of reaction tend away from this, the mass law may be invoked to account for a partial recovery. Obviously, if rates of reaction are of like magnitude, a mediator would be ineffective if the position of its potential curve were far above that of the more positive substrate system or far below that of the more negative substrate system. By the same token one may judge that the curve relating the efficiencies of different mediators to their potentials would have some such form as that indicated in Fig. 11.

That this view is adequate I do not assert; that efficiency curves comparable to that on the right of Fig. 11 have been found experimentally is a fact. For simplicity Fig. 11 is drawn for equimolecular quantities of reacting substrates. It may be adjusted to other cases. Dealing as it does only with a special feature of catalysis and that crudely, there is no occasion to labor the subject

FIGURE 11

the hydrogen of the substrate has been activated. Thus arises the designation of such an enzyme by any one of several terms implying that only the reduced substrate and preferably the hydrogen thereof is activated. What of the oxidized state of the substrate—a necessary part of a *system*? I know no evidence that the enzyme succinodehydrogenase activates only succinate and not fumarate although I also know no decisive evidence that this could not be true. There is a good reason to assume that both oxidant and reductant are "activated" (whatever that may mean) and until evidence to the contrary is forthcoming I see no reason to let our nomenclature be determined by the accidental course of research. Experimentalists will realize that only a little ingenuity is needed to follow a course of experiment diametrically opposite to that which has determined nomenclature. Had this happened historically there could be many terms with connotations antithetic to those that are current. When this is appreciated it will be realized that current nomenclature is not being adapted to even the most elementary parts of our evolving knowledge. When it is so adapted it is to be hoped that it will become both simple and expressive of elementary experimental facts.

I dislike to keep referring to the old hydrogen transport theory in the way that I shall now do and would not do so had I not read in this year of grace, 1939, a review setting forth matters that are "as dead as a door nail". Like Marley, the hydrogen transport theory may yet reappear, for it is quite evident that the protons in organic molecules occupy unique spatial and energy positions and, as illustrated by exchanges with deuterium, vary greatly in "escaping tendencies" that well may be modified by catalysts: Let us first deal with a very elementary conclusion that flows from the quantitative measurements of oxidation-reduction equilibria and from the measurement of ionization energies. The conversion of a mole of methylene blue to one of methylene white has been shown to require two electrochemical equivalents. This and estimation of the ionization constants lead to the description of the over-all process given below. A similar description can be given for the reduction of an N-substituted nicotinic acid amide as in coenzymes I and II.

In each case the simplest interpretation is that reduction involves two electrons and but one proton. Such a description is in no true sense a description of mechanism but only of those constitutional changes that are reflected in energy changes of specific kinds. Nevertheless the description represents relations that must be accounted for in any theory of the mechanism of the change. All that need be said further is that the good evidence for the involvement of two equi-

unduly. Distinctions are here important. This is not a theory of catalysis; it indicates but one part that thermodynamic relations could play in circumstances amid which such relations are applicable. It does not purport to deal with the fundamental problem of specificity in catalysis; it only indicates one way in which a limited sort of specificity can obtain.

Since the juncture of thermodynamics and kinetics is fraught with complications that I am not now prepared to discuss, I shall make but one more, unimportant comment on the model presented above. It is perfectly obvious that when we write a series of equations involving the interaction of catalyst and substrate and then the regeneration of the oxidized or reduced catalyst, we resort to a step-wise and cyclic process as a convenience of expression. What must be recognized in so doing is both the nature of the "chemical equation" and the simultaneity of the actual reactions. In my own mind this is obscured when the term "cyclic process" is used. To me Fig. 11 appeals as easing the description for it makes it easy to see how the state of the catalyst, in this case a mediator, can "stay put". Furthermore, it presents a possible process in a manner that calls for no terms other than those common to the main subject matter of this discourse.

While mentioning nomenclature I should like to comment here, as I have elsewhere, upon the part played by circumstances in determining nomenclature. Operating as we usually do in an oxygen atmosphere we find the oxidized states of many reagents, notably certain dyes, to be the more convenient. Then, if a dye is not reduced by a substance thought to be concerned in cell metabolism, but is reduced on addition of an enzyme, it is but natural to say that the enzyme has activated this substrate, *i.e.*, made of it an active reducing agent. In terms of the hydrogen transport theory

chief process at pH 7 - 12

valents and only one proton should, alone, suffice to raise the eyebrow of him who gives unqualified allegiance to the exclusive terms in which the hydrogen transport theory was first cast. The reviewer, mentioned above, referred again to the palladium hydroquinone experiment of Wieland as a key experiment. Here the essential claim was that "hydrogen" (unspecified in state so far as I can find) can be extracted from hydroquinone by palladium. Now that electrode potential measurements have permitted the precise calculation of the energy required for such a process within any range of specifications, it is quite evident that within all reasonable specifications so much energy would have to be expended to effect the process claimed that the probability of it occurring spontaneously is practically *nil*. Wieland's experimental observations were explained in other terms eight years ago by Gillespie and Liu (1931) and so far as I am aware their account has not been gainsaid.

On one or another of the following bases one could criticize the greater part of the current nomenclature that flows from the older views. The ideas expressed or implied are frequently naive and out of harmony with advanced knowledge. The nomenclature represents the tendency to generalize before the facts are in, and before theory is both clear and in harmony with facts. But the most disturbing and irritating aspect is the fact that this nomenclature is, in principle and excepting the multitude of its synonyms, so uncritically accepted that it is frequently with the utmost difficulty that one can find the raw facts between the lines of accounts of experimental work written in its terms.

In conclusion let me mention some matters that are not at all clear and toward the clarification of which our subject can make some contributions of secondary importance.

I cannot escape the recollection of my own surprise, during the early mapping of dye systems and their use in explorations of cell suspensions, that direct, indirect, and inferential estimates of the reductive tendencies of the living cell placed the general region of potential far distant from that of the oxygen-water system (1.23 volts at pH = 0). Parenthetically I may add that such gross relations appeal to me as more exciting than the minor ones with which our limited knowledge of cell components and cell states is ill prepared to deal. Since oxygen enters the economy of the living cell, the crudely defined but obviously negative potential at which cell reactions occur must mean that oxygen is not activated to release its full inherent intensity but enters gently by a controlled route. To put the first matter more concretely, it appears that the biocatalysts are not concerned with the half-reaction

$$O_2 + 4e + 4H^+ \rightleftharpoons 2H_2O$$
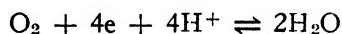
in the sense that the platinum catalyst at the hydrogen electrode is concerned with the half-reaction

$$H_2 \rightleftharpoons 2e + 2H^+$$

or

$$H_2 + 2H_2O \rightleftharpoons 2e + 2H^+_3O.$$
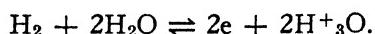
There is little or no evidence that the inherent oxidizing ability of molecular oxygen is made available for general oxidizing purposes as the inherent reducing ability of hydrogen is made available in varying degrees by the metal catalysts. Indeed, it is significant that there is as yet no indication of biocatalysts being used to make practical the oxygen electrode. This negative physicochemical evidence then supports the conclusion that has come by way of the partial isolation of the biocatalysts, namely, that the cell provides very special compounds uniquely adapted to combine with molecular oxygen and that the catalysis that makes oxygen available is through specific chemical reactions. It is not unlikely that the "activation" of oxygen then occurring is within a coordination complex and by a specific channel that removes the action from the general field. While I have not seen this stated explicitly, it is implicit in most of the modern treatments. Yet, curiously enough, statements of what little is known of this specific chemistry are often inconsistent with the implications of the language used to expound theory. Thus in the introductory part of a review of the highly specific actions of various

oxidases one finds the following statement. "The oxidases . . . appear to function by *activating oxygen* so that it will quickly oxidize a slowly auto-oxidizable compound." The italics are mine. I judge that an elementary student memorizing this introductory statement would be prepared to expect the oxidases to make available the destructively intense, inherent, oxidizing ability of oxygen. Yet, while the student memorizes this statement, the world about him runs smoothly on with the destructive potentiality of oxygen held in check. This much he can see for himself, however much he may be confused by what he is told.

Not yet adequately defined by experimental evidence, expressed quantitatively in the desired form, is the suggestion that the oxygen-hydrogen peroxide system, of much lower potential, is involved.

By use of the theoretical equation for the oxygen-water system, the potential of which, at constant pH, should vary as the fourth root of the oxygen pressure, it is possible to calculate the partial pressure of oxygen that should obtain in any system in complete equilibrium with the oxygen-water system. It turns out that such a calculation indicates that the oxygen partial pressure should be practically *nil* in cells maintaining any one of many dyes in the reduced state, provided equilibrium obtains. On the other hand the equilibrium prerequisite to such a calculation obviously does not obtain when, for example, oxygen is bubbled through a solution of reduced 2,6-dichloroindophenol without oxidizing the leuco dye. In such terms I redefined many years ago an old problem of anaerobiosis. It must be shown with reasonable surety that an equilibrium state is approached before reduction of a dye can be taken as evidence of oxygen exhaustion. I know of no way by which one could put this proposition to a rigid test. On the other hand, it is reasonable to use the velocities of the oxidation of certain dyes by oxygen and to draw this conclusion: when a bacterial culture, for example, shall have maintained in the reduced state for several hours or days a dye that is rapidly oxidized by air and has done so without there being any source of fresh supplies of oxygen, there must be the practical absence of oxygen. This reasonable deduction is of a kind very different from the answer expected for the frequently made question "what does partial reduction of a dye signify as to the oxygen concentration?" The form of this question suggests that the inquirer is thinking of what the partial transformation of an acid indicator signifies of hydrogen ion concentration.

The reasonable but not rigid evidence of the absence of oxygen in cultures of anaerobes focuses attention upon a now accepted principle obscured of old by emphasis upon the dominating impor-

tance of oxygen, namely that energies needed for the various uses of a cell can be obtained by chemical processes involving no molecular oxygen.

I have already mentioned the coupling of the energies of the oxidation-reduction process and proton exchange. We have had considerable interest in the empirically established fact that oxidation can inhibit certain hydrolytic enzymes. In some cases this has received potentiometric definition. This is a very remote approach to the problem of coupling oxidative and hydrolytic processes. So far as the matter has gone it involves a special chemistry that Hellerman is to discuss later during this Symposium.

Barron and Hastings (1933) have cited a case in which the catalytic activity of hemin is enhanced by the formation of the nicotine complex. They correlate this with an alteration of the potential of the system (see Fig. 12). It will be noted in Fig. 10 that a similar shift of potential occurs between riboflavin and its complex with a protein as yellow ferment. Whatever the final exposition of the alteration of catalytic activity may be in either case, and however much we disagree with theories offered, facts and correlations such as those cited by Barron and Hastings and by others demand respect. Therefore it would seem to be of ultimate practical value in the field of biochemistry as well as of current academic interest to study models in which energies of association between molecules are coupled with the energies of oxidation-reduction and of proton exchanges. My next paper will be concerned with this problem, as it is embodied in systems containing metalloporphyrins and nitrogenous bases. For the description of the data to be presented I shall extend "the same old equations".
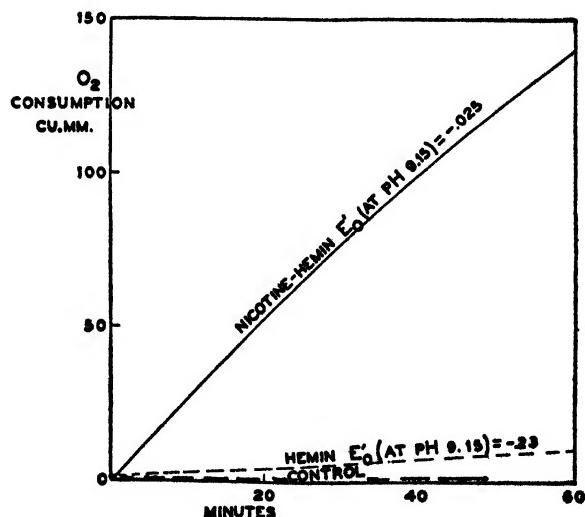

FIGURE 12

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DISCUSSION

Dr. Michaelis: It may be worthwhile mentioning that the fundamental equation referred to as Peters' equation was introduced by Van't Hoff and especially by Nernst. Bredig is responsible for the experimental test of the equation; Peters was a student of his, and performed the experimental test in Bredig's laboratory.

Dr. Clark: Yes, indeed; we are indebted to the whole Nernst school for the first of our modern formulations. Incidentally, however, if you will look back to the previous period you will find a considerable number of articles that have been neglected because, following the developments in the school of Nernst and Ostwald, the terms changed, and some of the older work has seldom been mentioned. Terminology and method of formulation very frequently set the trend of thought.

Dr. Barron: I would like to make the same plea as Clark has made regarding nomenclature. It seems to me that in no field is there such a state of confusion as in the field of oxidations. We have now for every kind of enzyme something like ten, twenty, or thirty different names, most of them given according to the fancy of the investigator, and the name "dehydrogenase", for example, had its origin in a faulty experiment. Since Lavoisier was the first to emphasize the importance of oxy-gen, and since it is the last element which takes part in the electronic exchange, we might retain the term "oxidase" in naming the entire enzyme component. I would like to join with Clark in a plea to simplify the nomenclature of the biological oxidation enzymes, and stick to the old name of oxidase whenever the entire enzyme is concerned. The components of the enzyme must be named according to their chemical constitution as soon as this is known.

Dr. Clark: I have only one comment on what Barron has said, and it is to point out the attitude which has developed since the emphasis first placed by Lavoisier on the participation of oxygen in life. You will recall that he not only reformed our entire view of chemical affairs, but that he also performed some very excellent biological experiments, and emphasized the fundamental importance of this newly discovered gas for life processes.

This emphasis upon the central position of oxygen is reflected in current books on physiology, where you find statements like this: "All life depends on oxygen". Now, anyone who has worked at all in the field of bacteriology knows perfectly well that there are many organisms which, so far as we now can tell, operate perfectly well in the entire absence of oxygen, getting their energy from internal oxidation-reduction processes involving no molecular oxygen. I have long felt that the anaerobic processes may in one sense be the simpler, because they are not complicated by the peculiar and unique series of mechanisms by which oxygen enters into metabolism.

This is no fundamental objection to what Barron has said regarding the retention of "oxidase", and I mean only to point out that we are still obsessed with the importance of oxygen. At times I have gone so far as to change the emphasis by saying that oxygen is a scavenger, which I think is going a little too far. Nevertheless, it serves to take oxygen out of a hypothetical position in which it was placed in the history of our subject by the early physiologists. Theirs was an attitude of mind which I think ought to be corrected, and it is for that reason that I urge students to think of oxygen as a scavenger, and a part of the garbage disposal system. I suggest that the entire perspective be reviewed before we go too far with "oxidase".

Dr. Fox: I would like to ask Clark two questions. First, does he care to interpret or to explain the fall in potential which occurs in bacterial cultures? Second, is he familiar with Clifton's technique of introducing non-toxic amounts of ferro-ferricyanide to steady the electrode potential?

Dr. Clark: The change in potential is a fact. There is a rough degree of duplication between

what one finds directly with the electrode and indirectly by the use of indicators that have been standardized by the electrode method. I think the simplest interpretation is that the electrode responds to the following sorts of systems. First, there are substrates in the cell that will not react readily. They are subject to "passive resistance", if that means anything; in the presence of the cell's catalysts, they are activated and begin to react, but they never reach a complete equilibrium state. Second, some cell components are now known to be electromotively active.

But you have a situation entirely different from what you have in studying acid-base equilibrium. In acid-base equilibrium you are dealing with the very rapid exchange of protons. Excepting slower changes that are involved in tautomeric rearrangements and barring certain slower reactions, such as the hydration of CO_2, you are always dealing with a state of equilibrium when dealing with proton exchanges. One can apply the thermodynamic method with assurance because there is an instant adjustment.

Now in the case of oxidation-reduction we have everything from cases like quinone-hydroquinone or the base-metalloporphyrins that react instantaneously, through cases like ascorbic acid, which we call sluggish systems, to those systems that have not yet been shown to affect the electrode.

With that background, with the recent revelation of certain components of living cells which are electromotively active and of others that are made so by cell catalysts, I think we can make a reasonable interpretation. When you put an electrode into a culture you may have present a small quantity of something carried over from previous cell action which sort of "half stabilizes" the potential. Then the cells manufacture small quantities of catalysts and activate the substrates to a small degree. The electrode responds to what may be called the vector sum.

But all the while you are dealing with superimposed changes with respect to time, not with a static and true equilibrium. In 1919 I gave up further intensive study of this subject, because I realized vaguely then what has become evident in more concrete form since: that we will not get far until we have isolated the components and studied them in isolation. We are dealing with a situation too complicated to analyze with any clarity. So I think at the present stage of the game the most important thing to do is to get hard at work on the isolation of components and the study of them in isolation.

Going on to Fox's second question, at the moment I do not recall the details of Clifton's use of the ferro-ferricyanide system and I do not have his paper at hand. In my early, and for the most part, unpublished work, I used several systems to poise the potential, among them that of ferricyanide, as well as several dyes. The outstanding result was that bacterial action is able so to mobilize the substrates of metabolism that they can reduce considerable amounts of oxidizing agents. The work thus performed may be of considerable magnitude. This is one of the best pieces of evidence that the observed changes of potential have a significance quite beyond the fortuitous. When we come to detail we are confronted not only with the general difficulty previously mentioned but also by special difficulties such, for instance, as this: Is ferro- or ferricyanide unable to penetrate the bacterial cell as it is unable to penetrate the red cell? If we compare the action of the ferricyanide and of other systems, may we not be in confusion until we know something of penetrabilities and can separate this and other specific factors from the general potential factor?

Nevertheless, let no fearful anticipations ever deter the explorer! The following quotation from Newton is apt in any general discussion of the present status of biological oxidation-reduction: "We are certainly not to relinquish the evidence of experiments for the sake of dreams and vain fictions of our own devising; nor are we to recede from the analogy of Nature, which is wont to be simple and always consistent with itself".

In the application of the method I have described in this paper we may be said to be playing in luck or playing in ill luck. There are some systems which I may call electromotively active. They affect the electrode. The potentials are stable. Other systems are quite inactive in the sense that they do not affect the electrode except in the presence of particular catalysts. I think at the beginning I pointed out that even in the case of our standard system, the hydrogen-hydrogen ion system, it is quite inactive, exhibits "passive resistance", until we introduce the catalyst, platinum or palladium-black. I would like to know if anyone here has any ideas, supported by experimental evidence, as to why it is that some systems are inactive and others active, electromotively.

I might point out that we cannot fall back on ionization uncovering the electrons in a molecule, for this reason: we can suppress ionization as we do in the case of many organic systems. Metalloporphins themselves do not give very satisfactory potentials, whereas, when coordinated with bases which we crudely think of as covering up the iron, they become electromotively active and the potentials are stable. Then there are familiar facts such as those pointed out by Shaffer which may bear on the subject too; that is, not all ionic systems that one would ordinarily expect to react come to equilibrium quickly. I would like to know if any-

one has any ideas on this matter, because if we have a theoretical basis of prediction we would not have to "cut and try" so much.

Dr. Barron: I quite agree that to establish the nature of electroactivity is a matter of the first importance, because a number of substances which act as mediators of electron exchange in biological oxidations are those systems which we call sluggish oxidation systems. Pyridine nucleotides, diphosphothiamine, and cytochrome-c belong to this group. If we assume that electroactivity depends on the degree of semiquinone formation, we have to find another explanation for systems where oxidation-reduction occurs with one-electron transfer. As Clark has pointed out, the metalloporphin alone, where the metal, at least in the case of iron-porphyrin, is in ionic form, behaves almost like a sluggish system. As soon as a nitrogenous base is added, the system becomes electroactive. We can say then that the addition of a nitrogenous compound had done something to break the passive resistance of the iron porphyrin compound. But why is it then that we have the same sluggishness when a protein like globin attaches to the iron porphyrin compound? In effect when globin is added to ferrous porphyrin, the nitrogenous complex thus formed, hemoglobin, is more sluggish than iron-porphyrin itself. The fact that Taylor in Hastings laboratory has shown lately that the potential of hemoglobin-methemoglobin can be established as soon as an electroactive mediator is added, shows that this system is a sluggish system. It is true that in that case the iron also is in an ionic form, according to Pauling and Coryell.

The cytochrome-c system is still more interesting from the electroactive point of view. Cytochrome-c, which is also an iron porphyrin protein compound, behaves as a sluggish system from pH 5 to pH 10. If one makes the solution acid, let us say pH 2, or extremely alkaline, pH 12 or 13, the system becomes electroactive.

What has happened to the nature of the combination between the iron and the protein, of course, we do not know. We are working on this phase of the subject in our laboratory in collaboration with Theorell, who is furnishing me with the cytochrome-c, but we have not reached any agreement because, according to him, the addition of hydrochloric acid does not destroy the nature of the linkage between the iron porphyrin and the protein. Therefore the possibility that the nature of the linkage was destroyed in that solution has not been proven.

I think in the cytochrome-c system we have the most clear example of these two kinds of system, sluggish and electroactive, and in the cytochrome-c system we have also the most clear example of the biological importance of the existence of these

two systems, the electroactive and the sluggish. Cytochrome-c in alkaline or acid solutions is very rapidly oxidized by molecular oxygen, while cytochrome-c in neutral solutions is not oxidized at measurable speed.

I would like to ask Clark's opinion on this question: Why are some systems sluggish and other systems electroactive? So far I have myself no answer to the question.

Dr. Clark: It is I who am asking for illumination. There is one very simple and very elementary aspect I think should not be forgotten when we are dealing with a system. As I pointed out in developing the fundamental equations, we are dealing with a difference in the chemical potentials, or, rather, a difference in differences. To put it in another way, the single electrode is responding to the impact, as it were, of the reductant and *also* the oxidant. So we cannot by the method at hand pick out the characteristics of an oxidant or the characteristics of a reductant *alone*. We are dealing with the two things that are acting; one can be considered a donor and the other an acceptor of the electrons. So we must not forget that our method is fundamentally concerned with both oxidant and reductant. This is plain and elementary. Nevertheless I bring it up because I have a feeling that the answer is going to come only when we know the characteristics of some particular oxidant alone, some particular reductant alone, and can synthesize what we know of those individuals to describe the resultant effect on the electrode.

You mentioned the semiquinones. I recognize clearly the contribution that Michaelis has made and that he is to discuss later. I wonder whether the one-electron jump of which much has been made is the final answer. It may be a contributory element, but I would not be at all surprised if exceptions were found and those exceptions would point to something more fundamental.

Dr. Michaelis: As regards the principles just mentioned, Barron has stated that in general those systems which easily respond to electrodes are also those of which the reductant is easily oxidized by molecular oxygen. That is often the case. But I must point out some exceptions in order to avoid a generalization of this principle. Take, for instance the ferrous ion. This is not oxidized at all by molecular oxygen. A slightly acid solution of ferrous sulphate is not autoxidizable; the same holds for ferrocyanate ion.

Dr. Gorin: This will not give much comfort concerning the question Clark brought up, but from a fundamental point of view these problems are very difficult; that is, the reactions in which electrons are exchanged are more difficult to deal with theoretically than those in which there is a smooth transposition of electrons from the old

bonds to the new ones as the groups approach. In the activated complex, according to the ideas of Eyring and collaborators, the electrons may be thought to belong neither to the old nor to the new bonds. The Eyring approach does not apply to oxidation reactions, however, since the smooth transposition of electrons from one atom to the other is usually theoretically impossible in these cases. Instead, the electron suddenly jumps from one atom to the other; the probability of jumping depends upon the distance between the two atoms concerned.

Dr. Preisler: In working with systems (such as ceric-cerous and thallic-thallous) which react with each other only very slowly, presumably because of unequal valence changes ($2Ce^{++++} + Tl^+ = 2Ce^{+++} + Tl^{+++}$), it was found that whereas each system alone gave stable reproducible potentials closely following the theoretical equations, these systems when mixed were unpoised and potentials read at different electrodes of the same or different metals differed widely. Thus, if to a mixture of cerous and ceric sulfates in sulfuric acid, giving a steady and theoretically correct potential, were added relatively small amounts of thallous and thallic sulfate, the potentials became immediately unpoised. Two active systems were then present, each trying to exert its own characteristic potential effect at the electrodes. If the proper catalyst (colloidal Pt or some Mn^{++} salt) were added, thallous reduced ceric until both systems were essentially at chemical equilibrium with each other and poised potentials were then read. This same, or a similar, factor may be operative in other cases. It seems possible that in many of the solutions which give unpoised potentials, the various component systems, oxidant-semiquinone, semiquinone-reductant, and oxidant-reductant, have not reached final equilibrium and more than one system is exerting an effect upon the electrode, thereby causing the erratic results.

Since the introduction of the concept of semiquinones, the question arises regarding the possible causes of deviations from the "two-equivalent change" theory in terms of which much of the older data have been formulated. From your own experiences, to what extent do you believe the deviations might be due to semiquinone formation, failure to establish true equilibrium, or to other causes?

Dr. Clark: In handling data there must go hand in hand an appreciation of the experimental circumstances and an appreciation of the value and limitations of certain methods of analyzing data.

First: I have yet to meet an organic compound that can be considered absolutely pure for electromotive force measurements. If the impurity is slow in reacting, there may not obtain a complete, simultaneous equilibrium. The effect may be most evident on the asymptotic branches of the titration curve.

Second: As we demonstrated with indigo, sulfonated to different degrees, a mixture of two or more reversible systems will give a titration curve departing markedly from that of the ideal $n = 2$ case.

Third: The equations ordinarily used are what are called "classical equations". Perhaps in the course of a titration made in a buffered solution of rather high salt content the change in activity coefficients may not be large. There will inevitably be some change and, if not measured, some estimate should be made of its relation to the cumulative experimental error. In some systems it should be given particular attention. For example, when methylene blue is reduced the structure changes from one of distinctly polar nature to one of symmetry without polar nature. We showed the large "salt effect" in this case, but in all that early work the purpose was to develop the main features of a system and we did not make an intensive study of the activity coefficients.

Fourth: Let me note a difficulty in the mathematical analysis of data that merely suggest and do not show clearly a departure from the ideal $n = 2$ curve. In recent years we have analyzed all our titration curves with the aid of the method of Reed and Berkson. This method provides an analysis that is as near to being impersonal as one can get. If the physical law being followed by the system is the law assumed in the particular application of this mathematical method, the inevitable experimental errors will appear as a slight scattering of the experimental points about a straight line. A *distinct* trend of the experimental points away from this straight line indicates that another law should be assumed. But it may also be found that a *slight* trend away from the rectified line is so as to leave one in doubt whether or not it represents a systematic error. Or to look at the matter in another way, may not the process of rectifying the sigmoid titration curve automatically select the part that comes nearest to fitting the postulated law when the actual departure from that law is so slight as to be obscured by the experimental errors? It is for this reason that I insist that scrutiny of the experimental circumstances and the mathematical analysis of data go hand in hand.

Of course it is true that analyses were made formerly without the scrutiny now demanded by the semiquinone theory. Indeed, I shall give Michaelis occasion to smile when I recall that we missed something in our early work by trusting that the $n = 2$ relation, found at one region of pH, would hold at all values of pH. Frequently we

determined the E'_0:pH curve with the method of mixtures and failed to make titrations at all values of pH. Because of such incompleteness the early work needs some reinvestigation.

It may be noted that Michaelis was wise in choosing for first treatment those cases in which the "spread" was wide. Such choice of favorable cases is essential in the beginning. If the attempt now be made to extend the theory to cover all cases, I suggest that proper consideration be given for both the sources of experimental error and for the advantages and limitations of some such method as that of Reed and Berkson which eliminates the personal factor from the mathematical treatment.

Dr. Michaelis: Our only hope in getting better experimental evidence with respect to the cases where there may or may not be a very small amount of semiquinone is if we could improve our magnetic methods, but unfortunately this method is not sensitive enough.

Dr. Baumberger: With respect to the difference between those compounds which quickly es-tablish a potential on the electrode and those which are more sluggish, I would point out that the ordinary method which is used is one in which the electrochemical reaction taking place at the electrode is minimized as much as possible. Either a null point method or a vacuum tube gal-vanometer is used.

There is another way of approaching the question, worked out by Müller and myself, namely, the use of the polarographic method for the meas-urement of oxidation-reduction potential. The potential is applied to the dropping mercury elec-trode and there is actually a considerable electro-chemical reaction taking place. It would appear that this would be a condition in which one would be more apt to avoid the difficulties that arise from the ordinary null point potentiometric method. I might add that in extending that idea we determined the potential of the quinhydrone system and obtained accurate E_h measurements at higher dilutions than by the ordinary method. We are also able to measure the polarographic ap-parent reduction potential of some compounds.

POTENTIOMETRIC AND SPECTROPHOTOMETRIC STUDIES OF METALLOPORPHYRINS IN COORDINATION WITH NITROGENOUS BASES

W. Mansfield Clark

The potentiometric study of heme was initiated by Conant and his students (1928-30). They noted among other matters the effect of added pyridine and gave for this an equation that is useful for an approximate description of the effect under very limited conditions.

Several years ago, in an attempt to approach the subject systematically, we took hold of what now appears to have been the wrong end of the problem, from the point of view of an experimentalist. We concentrated our first attention on heme in the absence of coordinating bases. Then Barron's (1937) excellent work showed that stable potentials are obtained only in the presence of one of several nitrogenous bases (including cyanide). To date neither he nor we have obtained wholly satisfactory potentiometric data for any metalloporphyrin in the absence of a coordinating base, although Barron's data for heme are adequate to "rough out" some of the characteristics of this system. In Fig. 1 are shown Barron's data on the relations between potentials and concentrations of added bases at constant degree of reduction and constant pH. Other aspects of his work will be mentioned later.

Taking our cue from Barron we turned to measurements of systems containing coordinating compounds and proceeded with the guess that something would come from a variation of metal and of porphyrin as well as a variation of the coordinating compound. Following that guess has proved both discouraging and profitable.

In the first instance I made a misjudgment. Believing that the hydrogenation of the vinyl groups in protoporphyrin to ethyl of mesoporphyrin would lead to more stable metalloporphyrins, as it does, I concentrated the attention of my students on the iron, cobalt, and manganese derivatives of *mesoporphyrin*. Progress was thereby delayed for, to our consternation, the reductants have such low solubilities as to restrict greatly the range of experiment. This discouragement was but the beginning. The several systems so far studied have properties that block crucial tests of principles at one point or another, yet, when the properties of one system block the experimental test of a crucial point, one may find another system better adapted to the test. Thus it now appears that a comprehensive view will have to be pieced together from the partial data of different
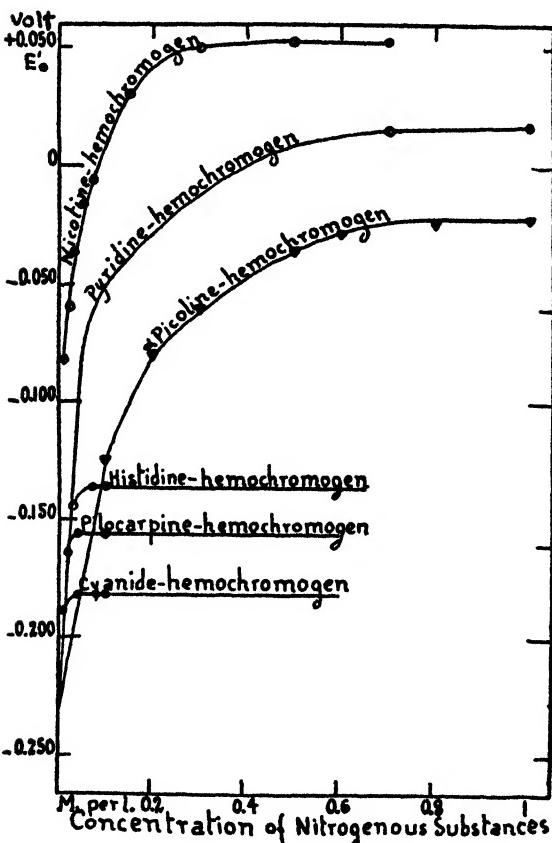
Fig. 1. Relation of potential to concentration of added nitrogenous base, at constant pH and constant degree of reduction. (After Barron, 1937).
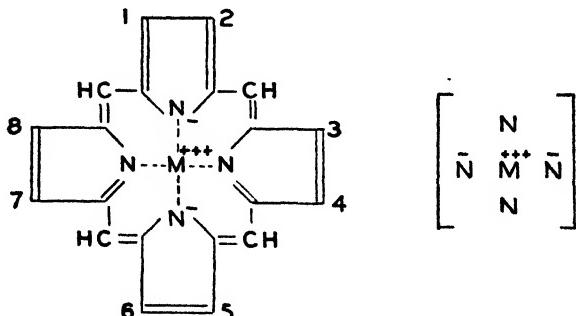
Fig. 2. Structure of metalloporphin from which are derived metallo derivatives of the following porphyrins.

Protoporphyrin IX: 1, 3, 5, 8-tetramethyl-2, 4-divinyl-porphin-6, 7-dipropionic acid.

Mesoporphyrin IX: 1, 3, 5, 8-tetramethyl-2, 4-diethyl-porphin-6, 7-dipropionic acid.

Hematoporphyrin IX: 1, 3, 5, 8-tetramethyl-2, 4-di(α-oxyethyl)porphin-6, 7-dipropionic acid.

Etioporphyrin I (syn.) 1, 3, 5, 7-tetramethyl-2, 4, 6, 8-tetraethyl-porphin.

Koproporphyrin I (syn.) 1, 3, 5, 7-tetramethyl-porphin-2, 4, 6, 8-tetrapropionic acid.

$M+++$ ≡ metal ion.

systems. Therein lies the profit of making several variations of components. Here I gratefully acknowledge the invaluable aid of Dr. Corwin of our Department of Chemistry in whose laboratory Vestling synthesized etioporphyrin I and koproporphyrin I and from whose laboratory have subsequently come supplies of ferrikoproporphyrin I. As I shall show, these materials have proved to be especially valuable.

Fig. 2 shows the structures of the porphyrins used.

The potentiometric measurements on part of which I report today were made by my students, John Taylor, Harrison Davies and Carl Vestling. Taylor, who is here today, is a good representative of this keen and hard-working crew. As you may judge by your acquaintance with the representative, all are good fellows who will not mind if I steal their thunder. [See Taylor (1937), Davies (1938) and Vestling (1938)]. I shall have to relieve them of responsibility for part of what I am to say, since recently acquired spectrophotometric data have changed my own views of certain matters. These views may change tomorrow. Indeed in no field have I said oftener, in jocular mood: "Don't make too many experiments".' Please regard what follows as a progress report. Much remains to be done.

Since current nomenclature is clumsy and misleading when its terms, derived from references to the blood pigment, are applied to compounds containing metals other than iron and containing

Table I

After Taylor (1937).
Demonstration that $n = 1$.
Relation of E_h to percentage reduction.
Cobalt mesoporphyrin nicotine.

Concentration of nicotine, 0.30 molar.
Concentration of total pigment, 0.00025 molar.
Titration with reduced phthiocol.
Temperature, 30° C.
Phosphate buffer, pH = 11.4 (approximately), $\mu = 0.45$.
Analysis of data by the method of Reed and Berkson.
$d = 0.08$ ml.   100 p.c. reduction at 7.84 ml.

y	y-d	Reduction	0.06011 $\log S_o/S_r$	E_h (observed)	E'_o	Deviations from -0.19923
ml.	ml.	percent	volts	volts	volts	volts
0.50	0.42	5.36	+0.07497	—0.12940	(—0.20437)	—0.00314
1.00	0.92	11.73	+0.05270	—0.14730	(—0.20000)	—0.00077
1.50	1.42	18.11	+0.03940	—0.15990	—0.19930	—0.00007
2.00	1.92	24.49	+0.02939	—0.16960	—0.19899	+0.00024
2.50	2.42	30.87	+0.02105	—0.17820	—0.19925	—0.00002
3.00	2.92	37.25	+0.01363	—0.18550	—0.19913	+0.00010
3.50	3.42	43.62	+0.00670	—0.19240	—0.19910	+0.00013
4.00	3.92	50.00	+0.00000	—0.19924	—0.19924	—0.00001
4.50	4.42	56.38	—0.00670	—0.20594	—0.19924	—0.00001
5.00	4.92	62.76	—0.01363	—0.21284	—0.19921	+0.00002
5.50	5.42	69.13	—0.02105	—0.22028	—0.19923	0.00000
6.00	5.92	75.51	—0.02939	—0.22864	—0.19925	—0.00002
6.50	6.42	81.89	—0.03940	—0.23885	—0.19945	—0.00022
7.00	6.92	88.27	—0.05270	—0.25221	—0.19957	—0.00024
7.50	7.42	94.64	—0.07497	—0.27620	(—0.20123)	—0.00200

Average   —0.19923

porphyrins other than those derived from the blood pigment, we have used a system that is presented best by way of illustration. The oxidized form of heme contains iron in the ferric state and protoporphyrin IX. This we call *ferri protoporphyrin* IX [see Fisher and Orth (1937) for the system of numbering]. By the same system the meanings of *cobalto mesoporphyrin* IX, *mangani mesoporphyrin* IX, *ferro koproporphyrin* I, etc., are clear. As a group these are called *metalloporphyrins*, and a particular system is designated by a name such as *iron koproporphyrin*, as an example. The coordination compound involving ferro koproporphyrin and pyridine is called *pyridine ferro koproporphyrin*. As a group such compounds are called *base metal-loporphyrins*, although this is tentative since allowance for coordination with other than bases (including cyanide) should be made.

Let us review quickly a few typical data on the number of equivalents involved in reduction and on the pH effect.

Table I is typical of a titration, the data of which, when analyzed by the method of Reed and Berkson (1929), yield evidence that one equivalent is concerned ($n = 1$). The symmetry of the titration curve together with the evidence that $n = 1$ speaks against any appreciable degree of polymerization of oxidant or reductant. Now contrast the cases exhibited in Tables II and III. In aqueous solution the nicotine iron protoporphyrins appear to be dimers while in 47.5 per cent

Table II

After Davies (1938).

The nicotine iron protoporphyrin system.

Titration of nicotine ferric protoporphyrin with reduced phthiocol in alcohol-buffer mixture at constant pH and constant nicotine concentration.

Demonstration that $n = 1$.
Solvent, 47.5 p.c. ethanol.
[Nicotine] $= 3.53 \times 10^{-1}$ molar.
[Total pigment] $= 1.44 \times 10^{-4}$ molar.
[Phthiocol] $= ca.\ 1.59 \times 10^{-3}$ normal.
pH of borate buffer $= 10.232$, $\mu = 0.05$.
Temperature, 30° C.
Analysis of data by method of Reed and Berkson.

y	$y \cdot d$	Reduction	0.06011 $\log S_R/S_0$	E_h	E'_0	Deviation from average
ml.	ml.	p.c.				
0.2	0.32	6.663	−0.0689	+0.0605	−0.0084	−0.0011
0.4	0.52	10.828	−0.0550	+0.0476	−0.0074	−0.0001
0.7	0.82	17.076	−0.0413	+0.0340	−0.0073	−0.0000
1.0	1.12	23.323	−0.0311	+0.0239	−0.0072	+0.0001
1.3	1.42	29.571	−0.0227	+0.0154	−0.0073	0.0000
1.7	1.82	37.900	−0.0129	+0.0053	−0.0076	−0.0003
2.1	2.22	46.230	−0.0040	+0.0030	−0.0070	+0.0003
2.5	2.62	54.560	+0.0048	−0.0124	−0.0076	−0.0003
2.9	3.02	62.890	+0.0138	−0.0214	−0.0076	−0.0003
3.3	3.42	71.220	+0.0237	−0.0314	−0.0077	−0.0004
3.7	3.82	79.550	+0.0355	−0.0433	−0.0078	−0.0005
4.1	4.22	87.880	+0.0517	−0.0589	−0.0072	+0.0001
4.4	4.52	94.127	+0.0725	−0.0800	−0.0075	−0.0002
4.6	4.72	98.292	+0.1058	−0.1057	−0.0001	+0.0074
				Average	−0.0073	

alcohol they appear to be monomers as deduced from the evidence that $n = 2$ in the first case and $n = 1$ in the second case. We shall refer again to this.

Barron showed very clearly that the potential of the iron protoporphyrin system in the presence of nicotine, pyridine and a few other bases, at constant concentration of base and constant degree of reduction, varies with pH according to the relation $-\Delta E_h/\Delta pH = 0.06$. In contrast, $-\Delta E_h/\Delta pH = 0$ in the case of the cyanide complex. We have confirmed both of these relations with the iron derivatives of proto-, meso-, hemato-, kopro- and etioporphyrins (see Fig. 3 and 4).

The relation $-\Delta E_h/\Delta pH = 0.06$ is evidence that for each electron that enters the reductant the reductant acquires one proton or the oxidant loses one hydroxyl group. Thus one proton is con-

cerned if $n = 1$ and two protons are concerned if $n = 2$. Another, remote possibility is that a group common to oxidant and reductant changes its ionization constant enormously as was illustrated in the case of the methylene blue system [See Clark, Cohen, and Gibbs (1925)]. Such a group common to oxidant and reductant might be one of the carboxyl groups of the porphyrin. But it is probable that they are completely dissociated in the range of pH concerned. Also the fact that a derivative of etioporphyrin, having no carboxyl groups, exhibits the pH effect shown in Fig. 4 directs attention to what we may call the coordination center represented in brackets in Fig. 2. The coordination of iron with etioporphyrin is one of the evidences disposing of the possibility that the metalloporphyrins are normal metallic salts of carboxylic acids.

Table III

After Davies (1938).

The nicotine iron protoporphyrin system.

Titration of nicotine ferric protoporphyrin with reduced phthiocol at constant pH and constant nicotine concentration.

Demonstration that $n = 2$.

[Nicotine] $= 2.9 \times 10^{-1}$ molar.

[Total pigment] $= 4.76 \times 10^{-4}$ molar.

[Reduced phthiocol] $= ca.\ 1.9 \times 10^{-3}$ molar.

pH of borate buffer $+$ nicotine $= 10.22$, $\mu = 0.05$.

Temperature $= 30°$ C.

Analysis of data by method of Reed and Berkson.

d (ml. of reducing solution oxidized by materials other than pigment) 0.83 ml.

100 p.c. reduction at $y = 8.2$ ml.

y	$y \cdot d$	Reduction	0.03006 $\log S_R/S_o$	E_h	E'_o	Deviation from average
ml.	ml.	p.c.				
1.11	0.28	3.797	-0.0422	$+0.0095$	-0.0327	
2.0	1.17	15.868	-0.02178	-0.0042	-0.0259	$+0.0000$
2.5	1.67	22.650	-0.01602	-0.0099	-0.0259	$+0.0002$
3.0	2.17	29.431	-0.0114	-0.0145	-0.0259	$+0.0001$
3.5	2.67	36.213	-0.0074	-0.0186	-0.0260	0.0000
4.0	3.17	42.994	-0.0037	-0.0223	-0.0260	-0.0001
4.5	3.67	49.776	-0.0001	-0.0259	-0.0260	-0.0001
5.0	4.17	56.557	$+0.0035$	-0.0296	-0.0261	-0.0002
5.5	4.67	63.339	-0.0071	-0.0333	-0.0261	-0.0002
6.0	5.17	71.021	$+0.0117$	-0.0373	-0.0256	$+0.0002$
6.5	5.67	76.902	$+0.0157$	-0.0414	-0.0256	$+0.0003$
				Average	-0.0259	
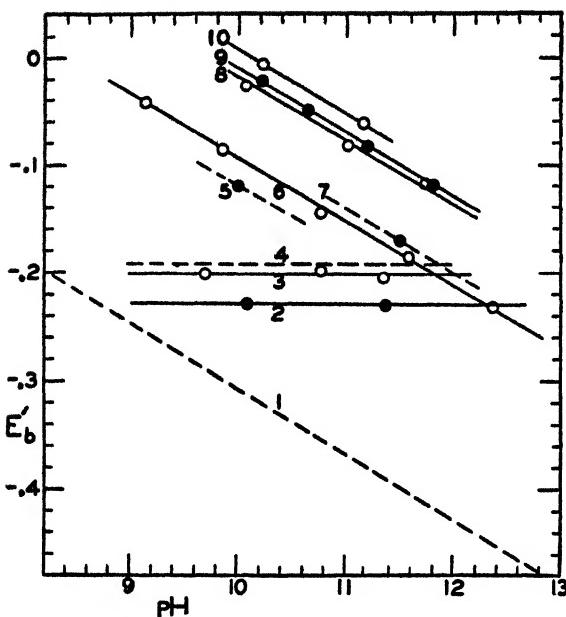
Fig. 3. Relation of potential, E'_0, at constant concentration of base and constant degree of reduction, to pH. (After Davies, 1938).

1. Iron protoporphyrin [estimated by Barron].
2. Cyanide iron mesoporphyrin.
3. Cyanide iron hematoporphyrin.
4. Cyanide iron protoporphyrin [after Barron].
5. α-picoline iron hematoporphyrin.
6. Pyridine iron mesoporphyrin in alcohol-water.
7. Nicotine iron mesoporphyrin in alcohol-water.
8. Nicotine iron protoporphyrin.
9. Pyridine iron hematoporphyrin.
10. Nicotine iron protoporphyrin in alcohol-water.

In contrast to the iron derivatives, the manganese and cobalt mesoporphyrins show no change of potential with pH whether in the presence of pyridine, nicotine or cyanide (see Fig. 5).

In the cases of the pyridine and nicotine iron koproporphyrin I systems the slope of each E'_0:pH curve changes from 0.06 in the more alkaline region and apparently approaches zero in the less alkaline region (see Fig. 6). This signifies the suppression of an acid ionization or its equivalent. On the basis of general principles treated by Clark and Cohen (1923) and Hall, Preisler and Cohen (1928) it can be shown that when the orientation of the inflexion of the E'_0:pH curve is that shown in Fig. 6, the change concerns the oxidant.

Perhaps the most interesting and certainly the most unique aspect of these systems is the reversible coordination between a base and the reduced and oxidized metalloporphyrins. It is generally acknowledged, and we have shown, that this is a quickly reversible process. In all the cases which we have studied the addition of base causes a positive shift of potential, all other conditions remaining constant. This direction of the change
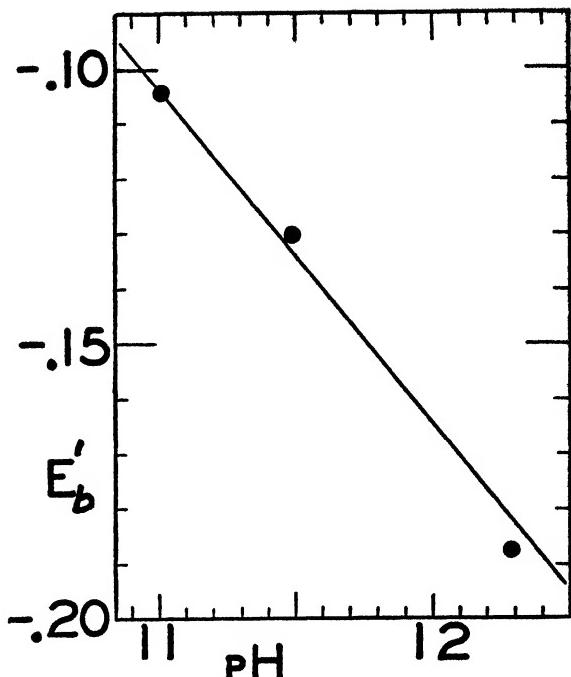
Fig. 4. Pyridine iron etioporphyrin. Relation of potential to pH. (After Vestling, 1938).
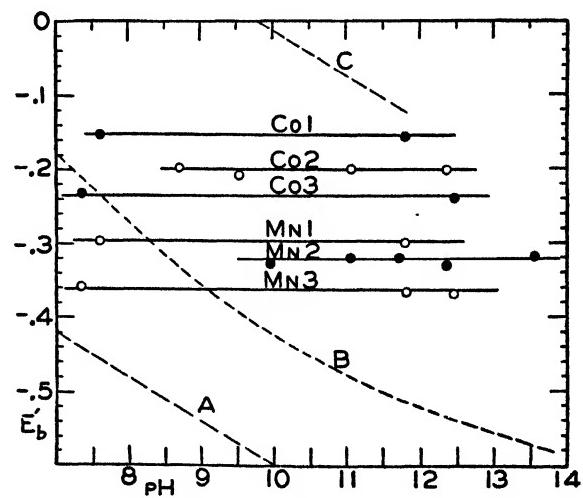
Fig. 5. Cobalt and manganese compounds. Relation of potential to pH. (After Taylor, 1937).

C   Pyridine iron protoporphyrin.
Co 1.   α-picoline cobalt mesoporphyrin.
Co 2.   Nicotine cobalt mesoporphyrin.
Co 3.   Pyridine cobalt mesoporphyrin.
Mn 1.   α-picoline manganese mesoporphyrin.
Mn 2.   Nicotine manganese mesoporphyrin.
Mn 3.   Pyridine manganese mesoporphyrin.
B.   Phthiocol.
A.   Hydrogen at one atmosphere.
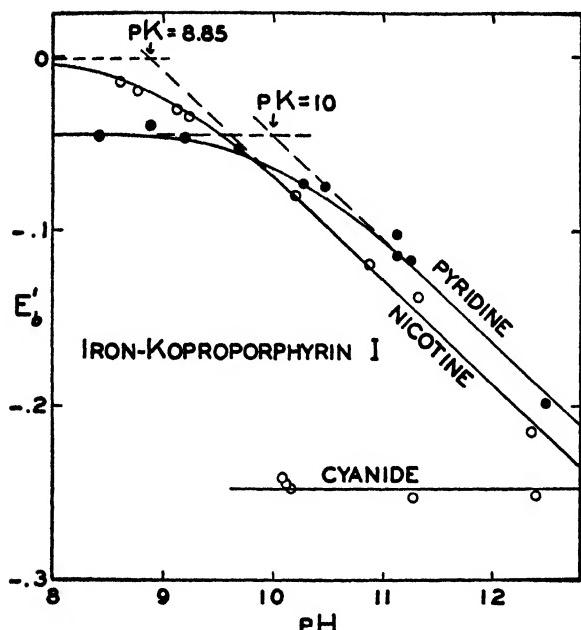
Fig. 6. Base iron koproporphyrin systems. (After Vestling, 1938). Relation of potential to pH.

of potential is due to a firmer binding between base and reduced metalloporphyrin than between base and oxidized metalloporphyrin. Qualitatively this explanation can be reached by the consideration of principles already developed for oxidation-reduction systems. It is embodied in equations systematically developed and then may be expressed as follows. If the numbers of molecules of base coordinating with oxidized and reduced metalloporphyrins are the same, the magnitude of the maximal potential change is determined by $(RT/nF)\ ln\ (K_o/K_r)$ where the constants K_o and K_r are the dissociation constants of the oxidized and reduced base metalloporphyrins respectively. These constants are defined by

$$\frac{[M_o]\ [B]^q}{[M_oB_q]} = K_o$$

$$\frac{[M_R]\ [B]^r}{[M_RB_r]} = K_r$$

That K_o is larger than K_r in the cases we have studied is confirmed in several instances by spectrophotometric measurements of K_o and K_r separately. If $q = r$, the potentiometric association curve will finally reach a plateau; if $q<r$, it will not.

Starting on the same sort of basis that Conant and Tongberg (1930) used in arriving at their

elementary equation, Clark, Taylor, Davies and Lewis (1938) gave more complete equations with which to approach an analysis of the association curves. These equations lend their aid better to graphic analysis when the curves are plotted with the abscissa made the logarithm of concentration of added base. Such charts strikingly reveal the fact that seldom has enough base been added to make certain the trend of the upper part of an association curve. With reservations on a matter still to be thoroughly investigated, we may assume that a plateau is reached when cyanide is the coordinating substance. If so, the same number of cyanide ions associate with oxidant and reductant $(q = r)$. In several other cases the potentiometric data may be described as well by the assumption that one more base enters reductant than enters oxidant. To indicate this the theoretical curves of Fig. 7, 8, and 9 are calculated with the assumption that two molecules of base coordinate with the reductant and one coordinates with the oxidant. This postulate should lead the curve to a final slope of $\Delta E_h/\Delta log\ [base] = 0.06$. A
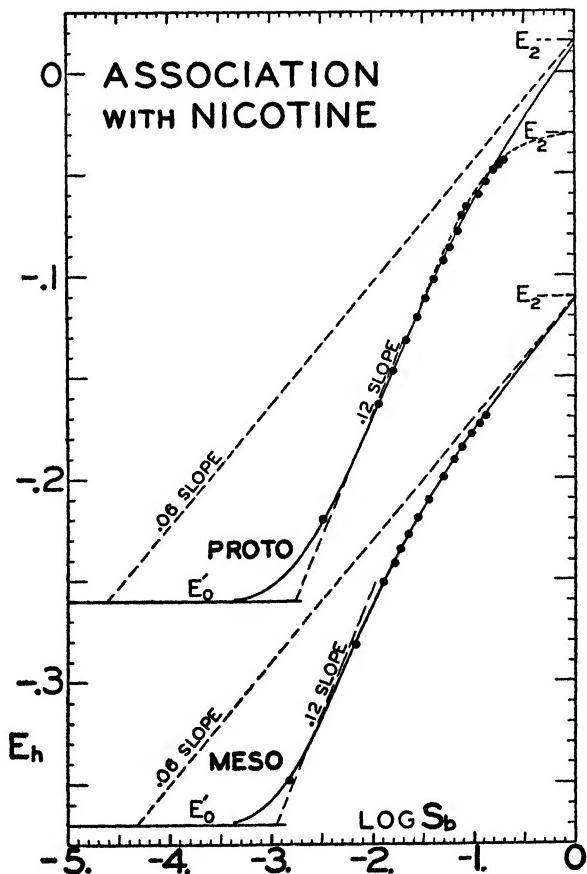
Fig. 7. Iron proto- and mesoporphyrin systems in water-ethanol solutions. (After Davies, 1938). Relation of potential to log concentration of added base.
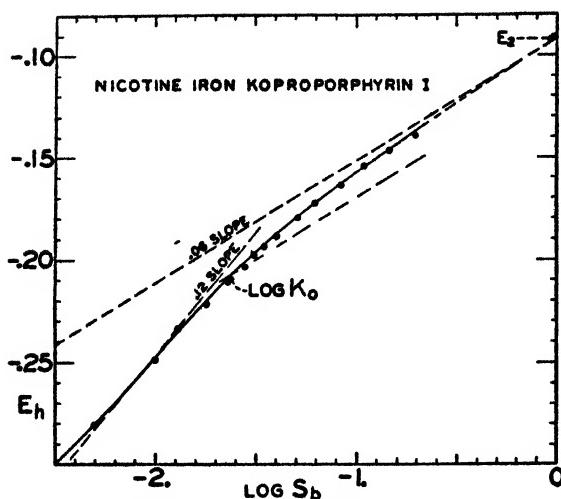
Fig. 8. Nicotine iron koproporphyrin. (After Vestling, 1938). Relation of potential to log concentration of added base.

criticism of this postulate will appear later. We are here representing as simply as possible what the face value of the potentiometric data suggests, although in Fig. 7 the experimental data for the protoporphyrin system are shown to fit the one assumption about as well as the other.

Neglecting for the moment the cases in which dimerization is evident, we may review the rather neat way in which Davies (1938) accounted for the relations so far presented. It is schematically given in part in Fig. 10. Here only the central portion of each structure is shown. The placements of signs refer only to origins since electric charges may not be located so definitely in a co-ordination compound. It is assumed that the metal ion replaces two protons of the pyrryl

groups and coordinates with the four pyrryl nitrogens. Artistically, the coordination number 6 is satisfied by the assignment of water molecules when not satisfied by base or hydroxyl ion.

The involvement of one electron in processes (2) (7) and (5) is demonstrated by Davies' data for some of the systems in 47.5 per cent ethanol, by Vestling's data for iron koproporphyrins in water and by Barron's results.

The involvement of H^+ in process (7) should give $-\Delta E_h/\Delta pH = 0.06$. This was demonstrated by Barron.

It is assumed that pyridine, and similar bases (B) can compete with coordinated water but not hydroxyl ion. Thus, if but one molecule of base enters in process (8) and two in process (3), $\Delta E_h/\Delta \log [B] = 0.06$; an interpretation permitted as is shown in Fig. 7, 8, and 9, but not proven. Also process (2) gives $-\Delta E_h/\Delta pH = 0.06$ when
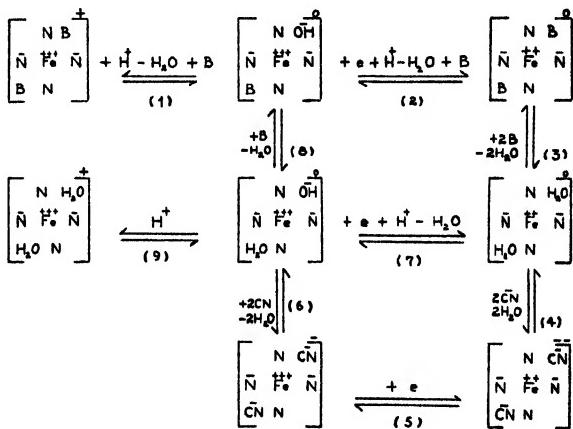
Fig. 10. Davies' (1938) tentative schema relating species in a base-iron-porphyrin system. See modification discussed in text.
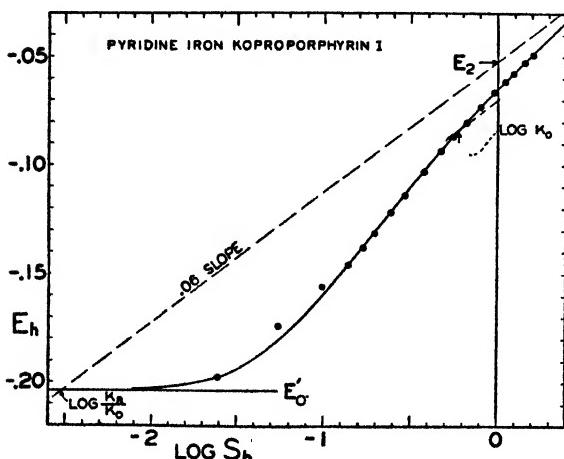
Fig. 9. Pyridine iron koproporphyrin. (After Vestling, 1938). Relation of potential to log concentration of added base.

(B) is constant. This is demonstrated clearly in Fig. 3. It is assumed that cyanide can compete with both coordinated water and hydroxyl ion. If the same number of cyanide ions (here assumed to be two) enter both oxidant and reductant, a plateau value is reached, as the data seem to indicate, and process (5) gives $-\Delta E_h/\Delta pH = 0$ when $[CN^-]$ is constant, as has been demonstrated (see Fig. 3). The ferri porphyrin should have an acid ionization constant (process (9)). One case of the measurement of this will be given later. Also the base ferri porphyrin should have an ionization constant, relating to process (1), and this constant might be expected to vary with the nature of the coordinated base or because of the simultaneous involvement of further addition of base as is postulated in the diagram. In either event Vestling picks up the individual acid ionization constants of nicotine ferri koproporphyrin I and pyridine ferri koproporphyrin I (see Fig. 6).

It is still uncertain whether the manganese and cobalt mesoporphyrins fail to coordinate with OH^- or whether the coordinating bases replace OH^- making these base metalloporphyrins comparable with the cyanide iron porphyrins. The first assumption seems the more probable. In any event $\Delta E'_0/\Delta pH = 0$. (See Fig. 5.)

We need not carry forward the discussion of this schema to the cases for which there is evidence of dimerization.

At present I see no reason to doubt some of the essential parts of this theory, but some of its detail may have to be modified. The chief doubts concern the number of molecules of base coordinating with the metalloporphyrin and the assumption of replacement of OH^- by base. The potentiometric association curves are not decisive. Recognizing this, Taylor, Davies and Vestling made valiant efforts to measure separately, by means of spectrophotometric data, the dissociation constant of one or another base metalloporphyrin, oxidized or reduced. They succeeded only in a few uncritical instances, because the "spread" between an adsorption curve of metalloporphyrin and that of the base metalloporphyrin is usually too small for adequate resolution by the instrument they used.

The independent test being crucial, I turned with some misgivings to the use of a General Electric "blocking-layer type" selenium cell. Having no current indicating instrument of desired range and sensitivity I employed a double White potentiometer in the arrangement shown in Fig. 11. Since the current from the cell is supposed to be in linear relation to the incident lumens only when the external resistance is zero, adjustment of the supplementary potentiometer, P, was made until the difference of potential across the terminals of the cell was zero. Then the fall of potential

across the fixed resistance R was measured by the White potentiometer. The substitution method was used. The response of the cell to the light passing through the solvent was measured in alternation with the response to the light passing through the solution. By having a double White potentiometer, and two potentiometers in place of the one shown by P, settings could be made and, on alternate substitution of solvent and solution, checked rapidly without resettings. Good stability of the light source, a 50 c.p. 6 v. automobile head-light lamp, was accomplished by floating two large 6 volt batteries in parallel across the input coming from balast lamps. Further detail may be left with the statement that the behaviour of this spectrophotometer was checked by the adsorption data of three colored glasses as determined by the Bureau of Standards. Assuming the Bureau's values to be absolute, I found the probable errors given below.

	Number of points reported by B. S.	Probable error of mean transmittance
Amber glass	8	± 0.0015
Blue glass	22	± 0.0015
Didynium glass	7	± 0.0026

With improvements made since these measurements were taken the reliability may be better, but this is not claimed at the present time.

In a system containing metalloporphyrin and base let there be the following species.

$M_a(OH^-)_h \equiv$ Metalloporphyrin containing a metal ions per molecule and h hydroxyl ions.

$M_a \equiv$ Species formed from above by addition of h protons.

$B_qM_a(OH^-)_h$ and $B_qM_a \equiv$ Species formed by addition of q molecules of base, B, to the above species. The equilibria are described by the following equations.

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Fig. 11. Diagram of main features of photoelectric photometer. Switches, dual connections, etc. omitted.

$$\frac{[M_a(OH^-)_h][H^+]^h}{[M_a]} = K_{h1} \quad (1)$$

$$\frac{[B_qM_a(OH^-)_h][H^+]^h}{[B_qM_a]} = K_{h2} \quad (2)$$

$$\frac{[M_a][B]^q}{[B_qM_a]} = K_1 \quad (3)$$

$$\frac{[M_a(OH^-)_h][B]^q}{[B_qM_a(OH^-)_h]} = K_2 \quad (4)$$

Also $K_{h_1}K_1 = K_{h_2}K_2 \quad (5)$

If conditions can be made such that only two of these species are present at the same time and if the molecular extinction coefficients of the two species differ considerably at some narrow band of the spectrum, it is possible to make spectrophotometric measurements of the distribution of the species as base is added or pH is changed.

It might appear that for this purpose a determination of the extinction coefficient of each of the two species separately is a necessary prerequisite. While this is desirable it is no more necessary than is the determination of the ends of an ordinary titration curve. We have successfully applied the method of Reed and Berkson to the rectification of the curves under discussion and to the calculation of the constants in the above equations. (See Fig. 12.)
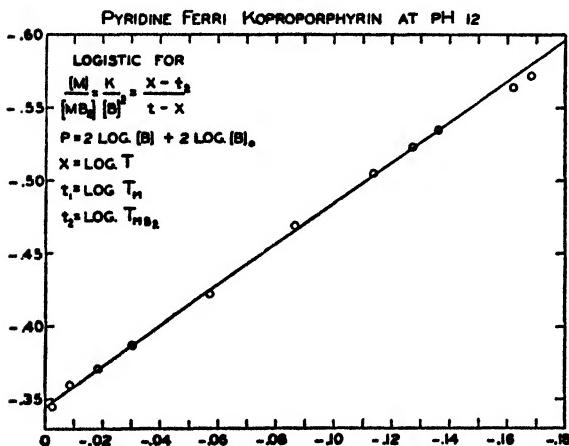
Fig. 12. Example of application of method of Reed and Berkson in rectifying relation between observed transmittance, T, and log concentration of added base. Data should give straight line the characteristics of which permit calculation of dissociation constant, individual extinction coefficients, etc.

First let me show in brief review a number of curves so calculated, and pertaining to incomplete series of measurements.*

While some of these data are open to different interpretations, those interpretations now seem to concern chiefly the question of the extent of dimerization. To date nothing has appeared that seems to invalidate the application of type relations developed by the study of the iron koproporphyrin system, and so attention will now be confined to this system, in which there is no evidence of dimerization.

* There were shown curves indicating that some of the base iron porphyrins in water solution are dimers and other curves suggesting change from a dimer to monomer. Interpretation is complicated by the possibility of one set of data being interpreted in two ways. To save space, discussion of these doubtful cases is withheld pending further work.
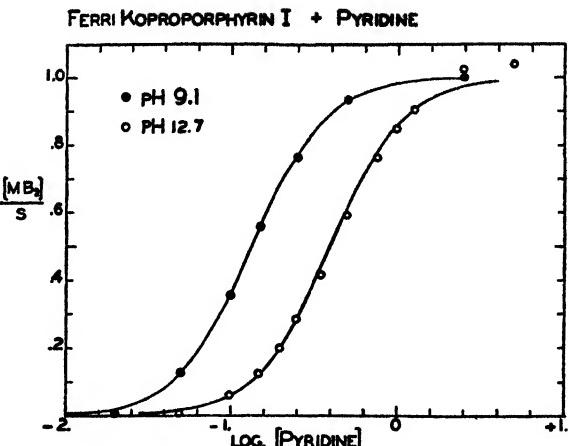
Fig. 13. Relation between log [pyridine] and $[MB_2]/S$, determined with spectrophotometer. $S = [MB_2] + [M]$. Curve theoretical for $M + 2B \rightleftharpoons MB_2$.

Fig. 13 shows data for the association of ferri koproporphyrin and pyridine at two different values of pH. The theoretical α curves are constructed on the assumption that two molecules of pyridine coordinate with one of metalloporphyrin.

Fig. 14 shows the spectrophotometric results when the concentration of pyridine is kept constant and pH is changed. The theoretical curve conforms to the assumption that one proton per molecule of metalloporphyrin is concerned. Since the results shown in Fig. 13 indicate that there is no difference between the numbers of pyridines when the association takes place above or below pH 10.3, we may assume the process $B_2MOH^- + H^+ \rightleftharpoons B_2M + H_2O$, or $B_2MOH^- + H^+ \rightleftharpoons$
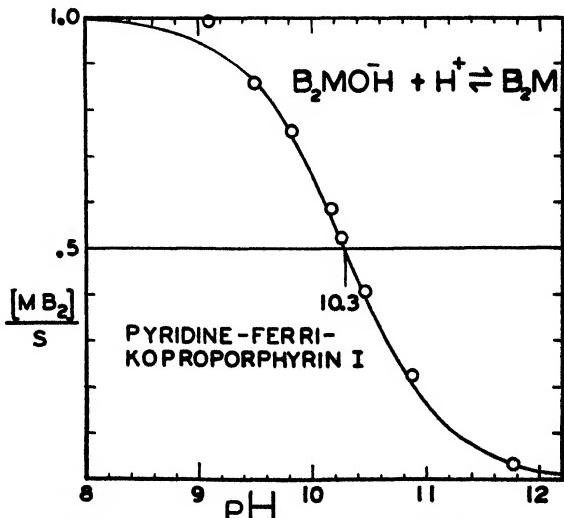
Fig. 14. Relation between pH and $[MB_2]/S$, determined with spectrophotometer. $S = [MB_2] + [B_2MOH^-]$. Curve theoretical for $B_2MOH^- + H^+ \rightleftharpoons B_2M$, or $B_2MOH^- + H^+ \rightleftharpoons B_2MH_2O$.
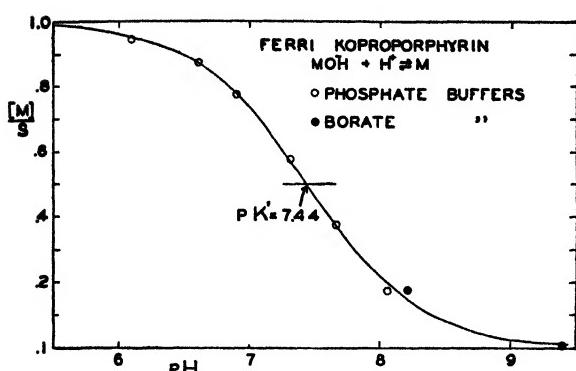
Fig. 15. Relation between pH and $[M]/S$, determined with spectrophotometer. $S = M + M\text{OH}^-$. Curve theoretical for $M\text{OH}^- + \text{H}^+ \rightleftharpoons M$ (or $M\text{H}_2\text{O}$).

$B_2M\text{H}_2\text{O}$. The dissociation exponent 10.3 is in substantial but not complete harmony with the value 10.0 shown in Fig. 6 by Vestling's potentiometric studies. That this ionization constant applies to the base metalloporphyrin and not to the metalloporphyrin itself is shown by two facts. First, as Fig. 6 indicates, the shift of the exponent with change of coordinating base is presumptive but not absolute proof. The second argument is revealed in Fig. 15. By reason of the solubility of ferri koproporphyrin itself we were able to study its solution at pH values as low as 6. A slight but distinct shift of absorption with shift of pH (see Fig. 16) made possible the collection of the data of Fig. 15 and these data indicate a dissociation exponent of 7.44 for ferri koproporphyrin in the absence of a coordinating compound.

Thus the acid dissociation constant of the metalloporphyrin is distinct from those of the base metalloporphyrins and the latter differ with the nature of the associating base.

What has been said so far concerns *ferri* koproporphyrin. If there be any change in pyri-

dine *ferro* koproporphyrin with change of pH it cannot be detected spectrophotometrically within the range of wavelength shown in Fig. 17. Here is shown the almost exact superposition of the absorption curves at pH 12.4 and 8.5. This confirms the deductions drawn from the potentiometric data.

There remains for consideration the association of pyridine and *ferro* koproporphyrin at constant pH. Here arise difficulties which are partly revealed with the aid of Fig. 18. There was chosen for examination a very narrow band with its center at mμ 548. The data relating log T to log [pyridine] are shown by open circles in Fig. 18.
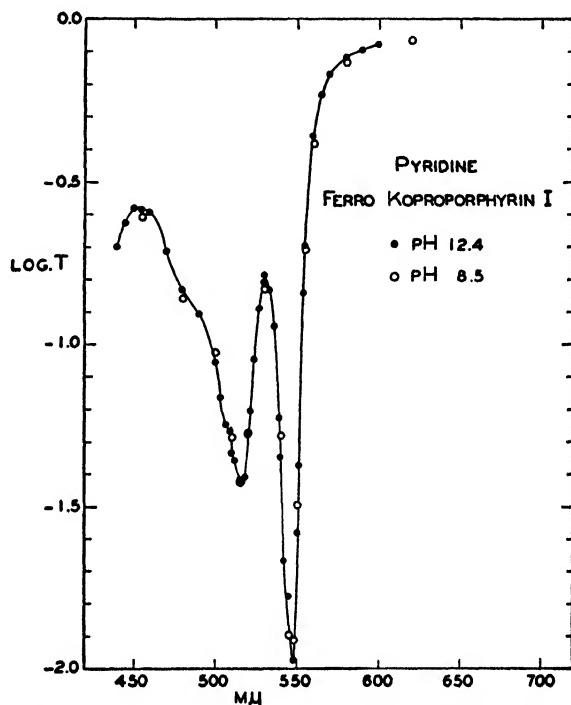
Fig. 17. Transmittance curves of pyridine ferro koproporphyrin showing independence of pH.

When resolved by the impersonal method of Reed and Berkson these data did not give a good α-curve. But there was noted a distinct shift of the peak of the absorption curve when the concentration of pyridine was increased over that which the preliminary calculation suggested should be enough to nearly complete the conversion of metalloporphyrin to base metalloporphyrin. This shift is shown by the inset at the right of the figure. Noting that the curves cross and provide an isobestic point, although perhaps one of limited significance, it occurred to me that by centering at this point I might avoid the confusion incident to some secondary change. Obviously more exhaustive study will have to determine the validity

Fig. 16. Transmittance curves of ferri koproporphyrin (in absence of base) as function of pH.
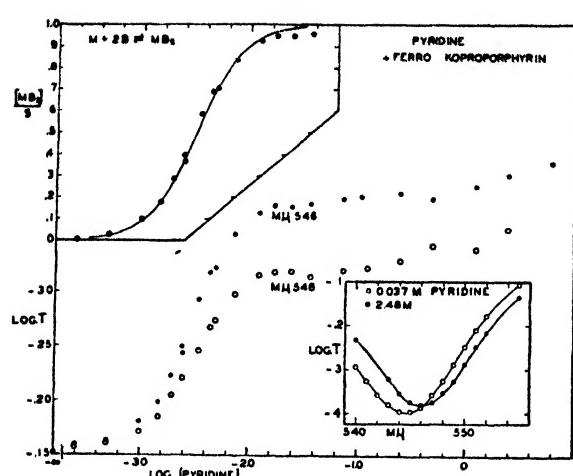
Fig. 18. *Center.* Original data relating log T to log [pyridine]. Measurements with center of observed band at mμ 546 and 548.

Right inset. Enlarged view of peak of absorption (minimum T) showing shift with addition of pyridine beyond amount expected to saturate metalloporphyrin.

Left inset. The "alpha-curve" calculated for $M +$ $2B \rightleftharpoons MB_2$ and data for the measurements at mμ 546.

secondary change takes place. Whatever the nature of this may be, if it be such that it makes r greater than q, in reality or in effect, the potential should continue to rise.

I am not prepared to say that the latter suggestion is the final answer to the apparent discrepancy between the potentiometric and spectrophotometric data, but at any rate it indicates that a final, precise description of all details of this system has been rendered difficult by hitherto unsuspected complications. The doubts concern affairs at relatively high concentrations of base. I see no reason to doubt the essential simplicity of relations exhibited for lower concentrations of base.

The evidence that $q = 2$ and that $r = 2$ in the first stage of association renders untenable that part of the theory exhibited in Fig. 10 which specifies that $q = 1$ and $r = 2$. Attending this revision is the following curious situation. If four of the six coordination bonds of iron are concerned with pyrryl nitrogen and two with pyridine, where shall we place the hydroxyl group postulated to account for the pH effect? I prefer at this moment to let experimental evidence settle such problems rather than to attempt their answer before all the data are in.

Up to a certain point, which concerns the behaviour of the ferro koproporphyrin at high concentrations of pyridine, the experimental data for the pyridine iron koproporphyrin system may be expressed by the equation which I placed on the blackboard. Since all the relations there embodied have been exhibited graphically there seems no need to discuss the equation in detail. What remains to be done is to establish complete consistency among the numerical values of the several constants. This will require intercalculations.

In closing this report I may make one further comment and this concerns a matter mentioned in my first paper at this Symposium. The systems under discussion exhibit very large differences in the free energies of association. These have been linked with the free energies of oxidation-reduction and acid ionization, thus extending the range of the integration of processes of different sorts. I like to think that although these are models of no particular practical importance, they serve to show something of the nature of the more complex integrations that occur when the living cell opens the channel for the simultaneous occurrence of different sorts of chemical action.

of this guess. At any rate a reexamination of the case with a band centered at mμ 546 yielded the fairly good α-curve shown in the left hand inset. The theoretical curve there drawn is for two molecules of pyridine per molecule of metalloporphyrin. The experimental points are those for the first section of the experimental curve only. There occurs either some secondary chemical change, such as the addition of more pyridine molecules, or some secondary physical change, that alters the absorption when the pyridine concentration becomes high.

In summary it may be said that the normal course of addition of pyridine to koproporphyrin is two molecules per molecule of metalloporphyrin whether the latter be in the oxidized or the reduced state. If $q = r = 2$, what shall be said of the potentiometric data that indicate $r = 2$ and $q = 1$? The answer appears to be twofold. The potentiometric data could not be carried to concentrations of pyridine high enough to be decisive on the value of q. Even a 1 molar solution of pyridine contains 8.05 ml. of pyridine per 100 ml. of solution and already appreciable corrections of pH have to be made even though pyridine at the high pH used is virtually a neutral body. Within the range examined potentiometrically the curvature of the association curve can be accounted for by association constants assigned on various assumptions. Secondly, the spectrophotometric data strongly suggest that after the first normal association of two molecules of pyridine with *reduced* metalloporphyrin a further,

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DISCUSSION

Dr. Michaelis: I wish to give some examples of my earlier work which, though not concerned with porphyrins, is at any rate concerned with iron complexes. Several years ago Friedheim and I measured potentials in complex iron systems such as pyrophosphate and oxalate and many other combinations. It was soon clear that reasonable results could only be obtained in working with a large excess of the complex-forming anion. Thus the condition is fulfilled that only one of the various possible complexes is present, namely that containing the maximum possible number of complex-forming anions. When a little iron salt is dissolved in a large excess of pyrophosphate or oxalate, you get a titration curve which fits very well with an ordinary univalent oxidation. According to the choice of the complex-forming anion, one may cover all potential ranges from the very positive ferric-ferrous ion system to the very negative pyrophosphate-iron system.

The case of a very curious complex, pentacyanoagno-ferriate (or -ferroate) is even more interesting. When the ferric form of this complex is reductively titrated, a very steep titration curve is obtained. It covers an enormous range of potential, and on analyzing this curve there is a little jump in the middle. The two halves are symmetrical, and each half consists of two successive overlapping univalent steps. Altogether we have four electrons involved in such a way that the first two overlap a little, and the last two overlap also, but the first pair is separated from the second pair by a distinct potential jump. How is it possible that such a compound can show four univalent steps? That there are several steps is confirmed also by observation of color changes (violet, soon turning to green and very gradually to light yellow).

Several years ago Davidson investigated this complex. He assumed that this complex is present in aqueous solution in some polymerized form. Our curves showing that there are four electrons involved seem to indicate a quadrimolecular aggregate.

My interpretation is a compound like this. (The dotted lines stand for hydrogen bonds.)
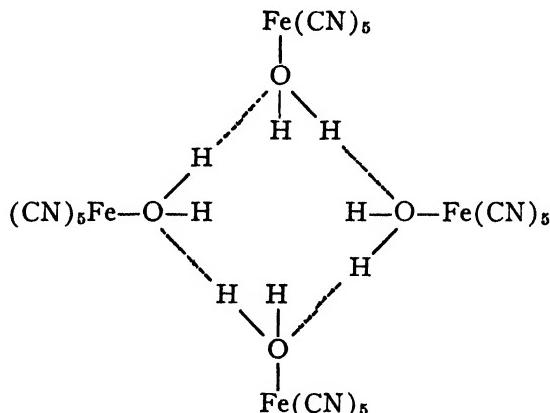
Considering the valence angles and spacial configuration (which cannot be dealt with in the short time of a discussion) such a structure seems quite possible. I wonder whether other polymerisations of iron complexes may find the same or a similar explanation.

Dr. Barron: Clark has determined the dissociation constant of the iron-porphyrin compound by his spectrophotometric analysis, and he uses and he has to use, of course, solutions in which he changes the hydrogen ion concentration by the use of buffers containing different anions. We have evidence that iron-porphyrin combines with phosphate and borate anions forming iron-porphyrin phosphate and iron-porphyrin borate. Although no change could be detected at all in the absorption spectrum of iron-porphyrin on addition of these buffers, the formation of complexes can be easily detected by the change of solubility. Whenever one takes a solution of iron-porphyrin, protoporphyrin, and adds it to a solution of phosphate the solubility is much greater than when one works in NaOH or borate. That made me suspect that several complexes existed and this suspicion was confirmed by potentiometric titrations. The potential of the iron-porphyrin-borate complex immediately led me to assume that one of the reasons for the difficulty in determining the equilibrium between hemoglobin and oxyhemoglobin was due to the fact that we were using different buffers. In effect, when to a solution of hemoglobin at pH 6.8 and an oxygen pressure of 5 mm., there is added bicarbonate, chloride sulfate, citrate or phosphate anions with the same ionic strength,

the percentage of oxyhemoglobin formed depends on the nature of the anion added to the solution, probably because the affinity of hemoglobin for oxygen is changed when hemoglobin combines with these anions. So it seems to me that when one is trying to determine the dissociation constant of iron-porphyrins using spectrophotometric data and one changes the hydrogen ion concentration of the solution by changing the ionic nature of these solutions, one has to take into consideration the possibility of complex formation between the metal porphyrin and the anion. This difficulty was also found in our work on the dissociation constant of cyanide-haemochromogens. We spent a lot of time making measurements on a buffer solution because we wanted to be sure of the stability of the hydrogen ion concentration of our solutions. Three or four months were spent in determining the dissociation constants in alkaline phosphate buffer solutions. Our results did not agree at all, because we were working with the iron-porphyrin-phosphate complex and our data would not agree with any equations we could formulate.

I would like to ask about the discrepancy between the spectrophotometric data and the potentiometric titration. There is no question that although the determination of the potential of the iron-porphyrin compound in sodium hydroxide solutions is extremely difficult, nevertheless, in something like 100 titrations I have done, there were some titrations which agreed satisfactorily with the usual oxidation-reduction equation involving one electron transfer. The titration is difficult because ferrohemin has a tendency to form a dimer compound which can be easily seen. From the spectrophotometric data obtained at Hogness's laboratory it had to be concluded that ferrihemin was the compound forming a dimer.

I have discussed the question at length with Young, a thermodynamic expert, who came to the conclusion that when there was a discrepancy between the spectrophotometric data and the potentiometric data, he would rely more on the latter than the former. So he is now studying the question himself in order to find out where is the flaw in the spectrophotometric data that we presented in studying the position constant of cyanide haemochromogen.

Clark has shown that the ferro-porphyrin-pyridine complex does not show a difference in absorption spectrum with increasing hydrogen ion concentration. Our observations agree with his. I would like to ask his opinion of the changed spectrum when one performs such experiments with the ferri-porphyrin-pyridine complex. Sidwell has shown that in such an event the spectrum of ferric-porphyrin-pyridine changes with the hydrogen ion concentration. That can be simply shown by mixing the ferri-porphyrin in alkaline solution with pyridine at pH 11; one gets a beautiful green compound. On the other hand, if one makes the solution at pH 8 the compound becomes a beautiful red. Why is there this change in the absorption spectrum?

Sidwell, working on the dissociation constant of the ferri-porphyrin-pyridine complex, did quite a number of experiments, but he met with much more difficulty than in the case of cyanide hemochromogen.

Dr. Clark: With regard to the specific salt effect, we have been quite aware of Barron's data on that subject. I confess we have not studied this matter as thoroughly as we should. Our first concern has been to "rough out", as it were, the main characteristics of the systems. In some of our cases we did not get the shift, with change in the nature of the buffer, that Barron got.

I showed the data with which we calculated the acid dissociation constant of ferri koproporphyrin, which has an adequate solubility in the neighborhood of pH 6 to 8. There, of course, we could have used other buffers, perhaps, but we had a limited quantity of material to work with and had to conserve every bit of it. As I remember it there were three points with borate and the rest with phosphate. The theoretical curve determined by all these points runs between one point for the phosphate and one for the borate and is close to the other borate points. In studying one of the base coordination compounds of iron mesoporphyrin or iron proto-porphyrin I repeated some of the measurements with buffers of different constitution. There was a slight difference, but in that particular case it was small. My students, who did the potentiometric work, could not confirm some of Barron's observations. Someone has to go over the work to see where the discrepancy lies. I have no doubt Barron is correct, and it may be that we have missed what he got because we have not controlled our conditions adequately. As Barron pointed out, there may be no difference spectrophotometrically with the borate and phosphate, whereas another method would show it.

Now as to the discrepancy between the potentiometric and spectrophotometric methods, I have no adequate answer. There is this, however, that must be said. I think in our application of the spectrophotometer to the study of equilibrium, we shall have to go back to the theoretical foundations of Beer's law. With current presentations I am not satisfied. One physicist tells me that I am quite safe in using Beer's law and another says not, and when I ask why not he says the answer is too complex. Also it must be realized that I have had to make the crude assumption that but two species are concerned.

Barron is right in saying that the potentiometric

data are the more trustworthy, but he should have emphasized that this is so only when the conditions are simple. As we have indicated by specific cases, it is possible to use any one of several sets of assumptions to fit a curve to the potentiometric data on association. Here there are too many constants for the certain evaluation of each one.

I may not have understood the other question. You asked, I believe, how we can account for the change of spectrum on shift of pH in the case of the pyridine metalloporphyrin. I showed a curve indicating that in the case of the pyridine iron koproporphyrin there is involved one proton or one hydroxyl ion and in the meso and proto compounds there are involved two protons or two hydroxyl ions. We have made use of the change in spectrum to calculate the acid dissociation constants.

Dr. Michaelis: May I suggest this proposition.

(1)

$$\begin{array}{ccc} N & | & N \\ & | & \\ N & | & N \end{array}$$

(2)

$$\begin{array}{ccc} | & N \text{-----} N \\ | & \\ | & N \text{-----} N \end{array}$$
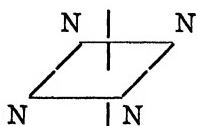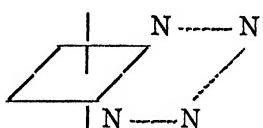
Usually, four corners of the octahedron (1) are occupied by the four N atoms of porphyrin and two are free for pyridine, etc. In competition of a very large excess of pyridine, only two may be occupied by porphyrin-N's, and the others by pyridine (2).

Dr. Fox: I saw some experiments comparing the effect of methylene blue with the effect of pyocyanine on respiration of tissue. Some experiments were done in phosphate buffer and some in other buffers. Those with phosphate showed a greater acceleration, whereas the same experiments under the same conditions with other buffer did not, and no explanation was given. I wonder if you have any explanation for that difference?

Dr. Clark: I can say only that methylene blue is a polar compound, and the cation of a strong base, whereas methylene white is not polar and is a weak base. Therefore a salt effect is to be expected and perhaps a difference between phosphate and other salts. However, when you come to the effect on glycolysis you have other factors entering. I don't think the salt effect would account for what you mention.

Dr. Barron: Clark has already emphasized that in determining the position constant of iron porphyrin with nitrogenous bases one has to take into consideration not only the ionic strength, the hydrogen ion concentration and the concentration of the reactants, but also the temperature. I think we have to emphasize more the influence of temperature. The temperature coefficient of the dissociation constant of these haemochromogens is extremely great, so great that by raising the temperature five or six degrees one can change the degree of dissociation. This has great importance from the biological point of view. The extreme ease with which these nitrogenous compounds combine with iron porphyrin (with such ease, in fact, that if one takes two nitrogenous bases and adds them to a solution containing iron porphyrins, one can displace one compound from another) makes the iron porphyrin compounds catalysts for many oxidations. It seems to me that Clark and I ought to have worked at lower temperatures in order to determine more precisely the dissociation constants. I feel that if we had worked at lower temperatures by increasing the affinity of the nitrogenous compounds to hemin it would have been easier to determine the dissociation constants.

Dr. Clark: When I repeat some of the spectrophotometric work I will have to set up temperature control. I am glad Barron has called my attention to the large temperature coefficient. I have been worried about it. It so happened that in the case of the koproporphyrins, I worked when the temperature in Baltimore was such that the laboratory was $30° \pm 1° $ C. The work was done in a closed dark room, where the temperature was rather constant, and I took care in every case to see that my solutions came to room temperature.

Dr. Baumberger: I would like to ask if the deviation of the spectrophotometric from the potentiometric measurement might be associated with the effect of polymerization on adsorption with respect to photometric measurement, whereas in the case of potentiometric measurement the polymerization might not have an effect? In studying the polymerization of pyruvic acid, B. L. Crawford found a change in the adsorption of ultraviolet light by pyruvic acid solutions of different concentrations and pH. If there are constituents that affect the degree of polymerization of metalloporphins it might affect the adsorption.

Dr. Clark: That is perfectly true. We utilize data of that sort. In some previous studies we also checked the evidence of polymerization by means of distribution coefficients between amyl alcohol and water solutions. And here Barron and

Hogness have made use of the shift in spectrophotometric adsorption to give evidence of dimerization. Of course you are extremely limited in what you can do spectrophotometrically. Ordinarily one has to assume that there are only two species to be dealt with. However, theoretically, if it be assumed that there is stepwise dissociation, or stepwise addition of base, and if the two constants of the individual steps are close enough together, there will result a curve as if the two steps were fused within certain narrow limits. The case is comparable to an acid-base titration, in which the two dissociation constants are nearly the same—a rare, but possible, case.

FREE RADICALS AS INTERMEDIATE STEPS OF OXIDATION-REDUCTION

L. MICHAELIS

Free organic radicals can no longer be considered as rarities. Since the first stable free radical was discovered by Gomberg in the form of triphenyl methyl, a great number of such radicals of various types have been prepared. Their common feature is the fact that they can be obtained only in water-free organic solvents and are unstable in the presence of water. It will be shown here that these properties are accidental for the group of substances investigated; that a great number of free radicals can exist even in aqueous solution; and that very often, and one might dare assert, even regularly, free radicals to a certain extent arise as intermediate products in reversible oxidation systems and are not necessarily molecules of restricted life-time, but can exist in true equilibrium with their parent substances. Organic oxidation-reduction systems are necessarily bivalent and the radical is intermediate between what is usually considered as the oxidized and the reduced form of the system. Among the properties of the free radicals there is one which should be pointed out with all emphasis from the outset: organic free radicals entirely lack that sluggishness of reactivity with respect to oxidation and reduction[1] with which many organic compounds are endowed. The equilibrium between a radical, its univalent oxidation product, and its univalent reduction product is established in solution with the same rapidity as the ionization equilibrium of an acid is established. Hence, it is impossible to prepare a pure solution of the radical or to measure its molecular weight by the customary methods. The unawareness of this fact caused all previous misconceptions about the existence and the properties of these radicals.

The oxidation or reduction of an organic compound to the level of a free radical requires a negligible amount of activation energy, whereas the activation energy in organic reactions otherwise may be very great, so great indeed that it makes possible the existence of many organic compounds which, purely thermodynamically speaking, should have no legitimate existence. If it be difficult to oxidize an organic substance by an oxidizing agent to a radical, it is not due to a high activation energy in the customary sense, but to the fact that the free energy of this reaction itself is very great, in other words that the radical is in such a case a very unstable compound. This consideration suggests the idea that the frequent occurrence of high activation energies in the oxidation or reduction of organic compounds is due to the fact that these reactions have to go in single univalent steps, and that what is designated as the activation energy is essentially the free energy to establish the intermediate radical. This radical represents the activated state through which the molecule has to go. If the free energy of establishing a radical is too great, the reversibility of oxidation-reduction is hampered. In such a case the concentration of the radical in equilibrium with its parent substance may be so small as to be the limiting factor of the rate of reaction. A catalyst, then, should have the property of entering into combination with the substance to be oxidized or reduced. This compound should be such that the establishment of the intermediate radical requires less free energy.

The study of free radicals is based essentially on three methods, potentiometric, magnetic, and colorimetric. First of all, these methods and their results will be discussed.

Potentiometric Methods

The standard method is the potentiometric titration, either oxidative or reductive. I shall present the matter considering only oxidative titrations; the other is quite analogous. A solution of the substance in question in its reduced state, in a solvent well buffered so as to maintain its pH during the experiment, is titrated with an oxidizing agent. The potentials, referred to the normal hydrogen electrode, are plotted against the degree of oxidation. This degree of oxidation can be measured in per cent of total oxidation; then its range runs from 0 to 100. Or, since we have to deal with two successive steps of oxidation, as will be shown, another suitable scale of degree of oxidation which we designate as the μ-scale, is such as to make its range run from -1 to $+1$. Then the point $\mu = 0$ is the midpoint, or 50 p.c. point, of the titration. Since the titration curves are symmetrical around the midpoint, it is advisable, when dealing with the mathematical aspect of the matter, to chose this midpoint as zero point. We may imagine that the experiment starts with the half-oxidized state and one experiment is performed by reducing it completely, another by oxidizing it completely, and the results of the two experiments are combined in one curve. Of course, we never perform an experiment in this way; it is just a manner of representation most suitable to make the functions symmetrical.

In what follows I shall describe the various experiments necessary to arrive at the characteristic properties of the systems, expressed in terms

[1] For reactions other than oxidation-reduction this is not necessarily true. The dimerization of radicals may sometimes be a reaction proceeding at a measurable rate.

of constants, and the method by which these constants can be derived from the titration curves experimentally obtained. I shall not give the proof for the correctness of the formulae applied for these calculations; they have all been described in previous papers. Rather, it will be shown how to handle the matter practically.

Suppose we have at hand such a titration curve plotted according to an experiment where the substance was dissolved in a suitable concentration, which is usually between 10^{-3} and 10^{-5} mols per liter, in some buffer solution. The first question is whether the curve represents a univalent system, a simple bivalent system without intermediate step, or a bivalent oxidation with an intermediate step, in other words, a bivalent oxidation consisting of two successive univalent steps. In a univalent oxidation, the curve at $30°$ C. should have the form[2]

$$E - E_o = 0.06 \log \frac{\text{p.c. of oxidation}}{100 - \text{p.c. of oxidation}}$$

$$= 0.06 \log \frac{1 + \mu}{1 - \mu}$$

Here E_o is the potential in the midpoint of titration, at 50 p.c. oxidation, or for $\mu = 0$; this constant is termed the normal potential for the particular pH (designated as E_h by Clark). In a bivalent oxidation without intermediate step we have

$$E - E_o = 0.03 \log \frac{\text{p.c. of oxidation}}{100 - \text{p.c. of oxidation}}$$

$$= 0.03 \log \frac{1 + \mu}{1 - \mu}$$

The curves may be calculated point by point as to their agreement with the one or the other formula. A preliminary calculation may be performed, considering only one or two characteristic points of a curve, as follows.

We measure the potential difference between 50 p.c. of oxidation and 25 p.c. of oxidation (*i.e.* at $\mu = 0$ and at $\mu = -\frac{1}{2}$). The absolute value of this difference may be designated as the index potential, E_i. This must be equal to the difference between 75 p.c. and 50 p.c. Otherwise the system is not an ordinary reversible bivalent system. If this symmetry is lacking, we may have to deal

with a mixed system; our compound may be impure, a mixture of two reversibly oxidizable substances of different potential range, instead of a pure compound; or the oxidized and reduced forms of the substance have different molecular sizes; one may be a dimeric compound, the other a monomeric compound. For the time being we neglect such occurrences, and assume that we have to deal with a single molecular species. For this case, in a univalent system $E_i = 28.6$ mv. (at $30°$ C.); in a bivalent system it is 14.3 mv. No other values of E_i can occur unless there is in a bivalent system an intermediate univalent step formed which is in equilibrium with the reduced and the oxidized forms. If there is such an intermediate step, $E_i > 14.3$, and there is no limit to its possible upper value. Suppose $E_i > 14.3$ in some experimental curve for a bivalent oxidation system. We have now to decide whether the intermediate form has the same molecular size as the other and hence is a free radical; or whether it is a polymerization product of the radical. The decision is based on the comparison of this curve with that derived from a similar experiment at the same pH, but using a different concentration of the substance to be titrated. If these two curves agree in all details, the intermediate compound has the same molecular size as the reduced or the oxidized form, and hence is a free radical. If E_i is not the same in the two curves, there is some polymerization product of the radical, either instead of the radical, or in equilibrium with the radical. Since polymerization is not a unimolecular reaction, the degree of polymerization must depend on concentration. So the following situations may arise and have in fact been encountered in practice.

(a) E_i is quite independent of the concentration. Then the intermediate form is certainly always the free radical.

(b) E_i depends on concentration, during the whole concentration range that can reasonably be used. Then, there is no definitely measurable amount of radical formed at all, and only the polymerized intermediate form exists. An example of such a case is phenanthrene quinone-sulfonate in acid solution, where only a dimerized radical exists. In very dilute solution, no intermediate form can be detected; as the concentration increases, the formation of a dimeric intermediate compound increases more and more. Since we have to deal with a bimolecular reaction, the results depend on concentration.

(c) There may be a certain range of low concentration in which E_i equals 14.3 independent of the concentration, but in a higher concentration E_i does depend on concentration. Then the intermediate form is a free radical which is in equili-

[2] The number 0.06 is set for $RT/F \cdot \log_{10} e$. Its precise value at $30°$ C. is 0.06001; in what follows, 0.03 stands for $RT/2F \cdot \log e$.

brium with its dimerized form. Such a case is realized in phenanthrene quinone-sulfonate in alkaline solution, and in lactoflavin over a wide range of pH.

Let us begin with the simplest case, where E_i is independent of the concentration. In this case we can derive from E_i the following magnitudes:

The equilibrium constant of the reaction

$$2S \rightleftharpoons R + T$$
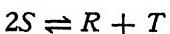
This reaction may be termed the dismutation of the radical. The three successive steps of oxidation are designated R (reduced form), S (semiquinone, or free radical form), T (totally oxidized form). The equilibrium constant is

$$\frac{[S]^2}{[R][T]} = k = \frac{1}{\kappa}$$

k is the semiquinone-formation constant. Sometimes it is more convenient to use its reciprocal, κ, the dismutation constant. The constant k can be calculated from E_i according to the equation

$$k = (10^{E_i/0.06} - 3/10^{E_i/0.06})^2$$

Furthermore, we can derive the maximum amount of the radical that is formed during the titration. This amount has its maximum, of course, in the midpoint of titration, at $\mu = 0$. Designating the total amount of the substance by a, that of the radical by s, then

$$\left(\frac{s}{a}\right)_{maximum} = \frac{\sqrt{k}}{2 + \sqrt{k}}$$

Furthermore, we may ask the question, at which point of the titration does the potential E equal E_1, the normal potential of the first step, i.e., at which point does the concentration of R equal that of S?

The answer is: E equals E_1 when

$$\mu = -\frac{k-1}{2k+1}$$

In the same manner the normal potential of the second step, E_2, where $[S]$ equals $[T]$, is that point of the curve, where

$$\mu = +\frac{k-1}{2k+1}$$

The normal potential of the whole system, or the mean normal potential, E_m, which is the normal potential in the customary sense, for the given pH (in Clark's sense E_h), is of course in the midpoint of titration, i.e., when $\mu = 0$.

According to the value of k, the curve may or may not show a jump around the midpoint of titration, which indicates two distinctly separate steps of oxidation. In other words, sometimes there is only one point of inflection, just as in a system without an intermediate step, at $\mu = 0$. Then the two steps greatly overlap. The difference from a simple curve without an intermediate step is then only that it is steeper; $E_1 > 14.3$ mv. Or, there may, in addition, be two lateral points of inflection. They occur only if $k > 16$. The location of the two lateral points of inflection is at those two points where

$$\mu = \pm\frac{1}{2}\sqrt{\frac{k-16}{k-4}}$$

It can be directly read from this equation that lateral points of inflection do not exist whenever $k \leqq 16$, since then the square root is no longer a real magnitude.

These equations imply much more valuable information than can be seen at first sight. I should like to point out some examples.

As we see, the minimum value of E_i is 14.3 mv. It occurs when there is no intermediate step at all. Suppose we have obtained this value in an experiment. Considering all possible errors such as slight change in pH during the titration, imperfect stability of the substances involved, possibly also slight changes of liquid junction potentials during the period of the experiment, etc., we may say that the limit of error, even in the very best cases, is no better than something like 0.1 millivolt, and is, in fact, usually greater. Let us assume that the true value is 14.4 and that this is within the limits of error of the experiment undistinguishable from the possible minimum value 14.3. Assuming 14.3, we obtain $k = 0$ precisely; no intermediate form exists at all. Assuming 14.4, what is the semiquinone formation constant, k, and what is the maximum fraction of semiquinone, $(S/A)_{max}$? The result of the calculation is

$$k = 0.00026$$

$$(S/A)_{max.} = 0.016$$

Hence, 1.6 per cent of the substance is present as a radical in the midpoint of titration. If an experiment indicates that no measurable amount of radical arises, it really means, assuming the
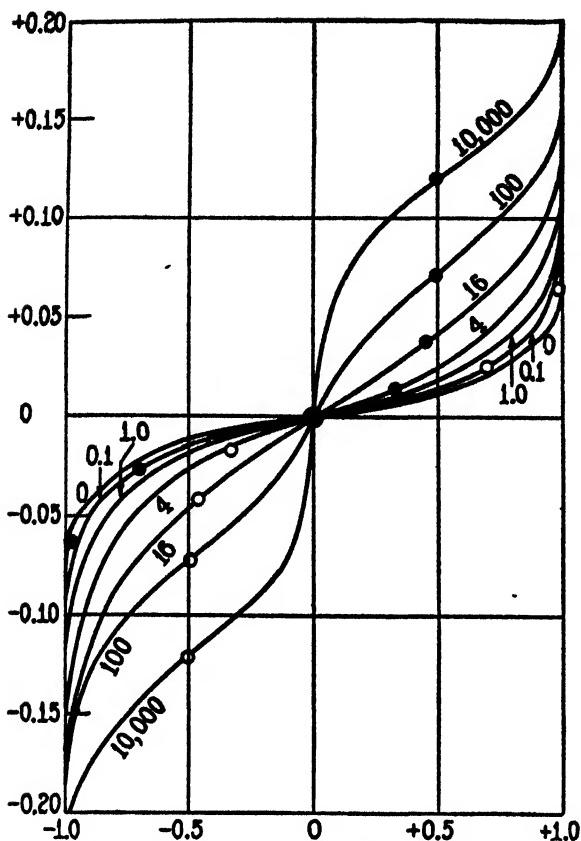
Fig. 1. Two-step titration curves involving formation of semiquinone radical. Abscissa: μ. $\mu = 0$ means 50 per cent of the total oxidation; $\mu = 1$ means 100 per cent of the total oxidation. Ordinates: $E - E_m$, the potential, referred to the mean normal potential E_m, in volts. Each curve holds for the value of k (semiquinone-formation constant) as indicated. White circle: that point of the titration curve where $E = E_1$ (the normal potential of the lower step). Black circle: that point where $E = E_2$ (the normal potential of the higher step). The black and white circle in the center belongs to the curve for $k = 1$; here E_m, E_1 and E_2 coincide at $\mu = 0$. White circles are on the left side for curves with $k > 1$; they are on the right side when $k < 1$. Both the white and the black circle in the curve for $k = 0$ belong actually to a curve for k intermediate between 0 and 0.1. When k is precisely 0, the circles would lie at $\mu = \pm 1$ and at potential $\pm \infty$. The lateral points of inflection begin to appear only when $k > 16$.

above limit of error, that not more than 1.6 p.c. of the substance is present as a radical in the midpoint of titration. The method is not sensitive enough to distinguish $(S/A)_{max.} = 0$ from $(S/A)_{max.} = 0.016$. This fact has an important bearing on the discussions to follow. We shall endeavor to show that certain properties of the reversible systems are due to the presence of radicals, and that very small amounts of radical are sufficient to account for the effect wanted. If we have other evidence for such an assertion, the

fact that the titration curve shows no intermediate step is not sufficient to discount any evidence otherwise obtained. It furthermore shows that all titration curves published which seem to show no intermediate step are subject to the same objection. These curves prove that *in maximo*, not more than a few per cent of the substance can be present as a free radical during the titration, but they do not prove that there is no radical formed at all.

Under ordinary conditions, a semiquinone formation constant smaller than about 0.01 cannot be measured by this method and cannot be distinguished from 0. This is just an insufficiency of the method, but there is no more reason to consider such a constant as insignificant if it is smaller than 10^{-2}, than it would be, say, for an ionization constant. The lack of any method of measuring small semiquinone formation constants is a great handicap at the present time.

The General Potential Equation

After evaluating all the characteristic constants of the system one should keep in mind that they have been derived only from the midpoint potential and from two more single points of the curve, the potentials at 25 p.c. and at 75 p.c. oxidation. The justification for the assumptions underlying these calculations should now be checked by testing the shape of the whole curve. This can be done by applying an equation expressing generally the potential in terms of a set of suitable variables and constants such as are independent of each other yet sufficient to characterize the system. The most amenable form of the equation is

$$E - E_m = (RT/2F) \ln \frac{1 + \mu}{1 - \mu} + (RT/2F) \ln \frac{\sqrt{1 + \gamma(1 - \mu^2)} + \mu}{\sqrt{1 + \gamma(1 - \mu^2)} - \mu}$$

where γ stands for $(4 - k)/k$ or, what is the same thing, $4\kappa - 1$. E is the potential in general, E_m is the potential at 50 p.c. oxidation, at $\mu = 0$. This equation shows the symmetry of the potential curve around its midpoint. It is an odd function, since reversing the sign of μ reverses the sign of $E - E_m$ without changing its magnitude. The radical formation constant appears only in the second logarithmic term, and only in the form $(4 - k)/k$. If k approaches zero, no semiquinone is formed at all. γ becomes much larger than all the other magnitudes in the second term, and this term approaches zero. Hence, the first logarithmic term is the one which holds when no intermediate form exists.

As long as free radicals as intermediate steps of oxidation-reduction were considered as an exceptional occurrence in a few dye-stuffs, it was not necessary to emphasize the existence of the second logarithmic term. As the list of dyes forming intermediate radicals has been increased more and more, it is quite unbelievable that there should be a sharp line between such dye-stuffs with intermediate radicals and without. The difference can be considered only quantitative. The second logarithmic term may be sometimes large, sometimes small, and even negligibly small, but perhaps will never be strictly zero. I dare say that among all the well investigated reversible dye-stuff systems only the thiazines and oxazines have shown so far no distinct evidence for forming an intermediate radical at any pH. There can be little doubt that this is a quantitative difference, not a qualitative one.

When $k = 4$, then $\gamma = 0$, and the second term becomes

$$\frac{RT}{2F} \ln \frac{1 + \mu}{1 - \mu}$$

which equals the first term. The potential curve, then, has the same form as that of a univalent oxidation,

$$E - E_m = 0.06 \log \frac{\text{p.c. oxidation}}{100 - \text{p.c. oxidation}}$$

containing the factor 0.06 instead of 0.03. The occurrence of this particular shape of the curve has been realized for a great number of dye-stuff systems at a certain pH. There is no chance of confusing this curve with a real univalent potential curve in spite of its equal shape since during the titration the intermediate form manifests itself by its characteristic color. Since $k = 4$, the maximum amount

$$(S/A)_{\text{max.}} = \frac{\sqrt{4}}{2 + \sqrt{4}} = 0.50$$

So, 50 p.c. of the substance exists in the form of the free radical in the midpoint of titration and cannot escape observation whenever its color is characteristic, which has been the case without exception so far.

So much about the interpretation of a single titration curve at a given pH.

The next task is to titrate the same dye-stuff at varied pH. The shape of the titration curve will in general vary according to pH. For each pH the various characteristic constants are computed according to the methods just described. Finally a summary of the results is presented best by a graph plotting the three normal potentials, E_1, E_2, E_m, against pH. These three potentials are interdependent in such a way that for each pH

$$E_m = \frac{E_1 + E_2}{2}$$

E_1 may be either lower than E_2, or higher. If the order is $E_1 < E_m < E_2$ we may speak of the natural order; if $E_1 > E_m > E_2$, we may speak of the reversed order of the three normal potentials. There will, in general, be one pH where $E_1 = E_m = E_2$, a point of intersection of the three normal potentials.

The relation between the radical formation constant k and the differences of the three normal potentials at a given pH is

$$E_2 - E_m = E_m - E_1 = 0.03 \log k$$

or

$$E_2 - E_1 = 0.06 \log k$$

Hence, at the pH of the point of intersection where $E_1 = E_m = E_2$,

$$k = 1$$

and

$$(S/A)_{\text{max.}} = \frac{\sqrt{1}}{2 + \sqrt{1}} = 0.333$$

At all pH's where there is the reverse order of the normal potentials, $k < 1$, and $(S/A)_{\text{max.}} < 0.333$.

The limits of error in drawing the E_m curve are never appreciable in a reversible system provided the electrode responds easily and reproducibly to it. The limits of error in E_1 and E_2 are just as small when $k > 1$, but become greater when k becomes much smaller than 1, because then an error even in some tenth of a millivolt in the index potential may appreciably affect the precise location of E_1 and E_2.

Considering the course of each of the three curves, it is essentially governed by the rules established by W. M. Clark, which, for the present purpose, may be summarized as follows:

In a univalent oxidation-reduction system, as represented by the E_1 and E_2 curve, the slopes of the curve in each of their approximately rectilinear sections can only be 0, or 0.06 volt per pH unit. In a bivalent system, such as is represented by the E_m curve, the slope may be 0, or 0.03, or 0.06, or sometimes 0.09 volts per pH unit. The rectilinear sections are connected by bends, and
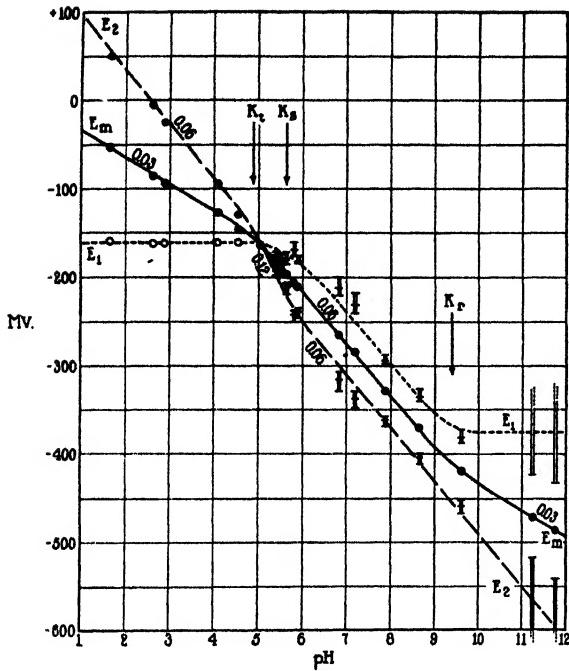
Fig. 2. Example of a cationic semiquinone. The three normal potentials of pyocyanine, plotted against pH. E_m = mean normal potential. E_1 = normal potential of the lower step of oxidation. E_2 = normal potential of the higher step of oxidation. At the crossing point, $k = 1$; to the left, $k > 1$; to the right, $k < 1$. K_t, K_s, K_r, bends due to acidic dissociation constants of the totally oxidized, the semi-oxidized, and the reduced form of the dye. To the left of the crossing point, $E_1 < E_m < E_2$, to the right, $E_1 > E_m > E_2$.

the intersection of the tangents occurs at such pH which equals a pK of one component of the system. ("K" is used for an acidic dissociation constant, or ionization constant.) If the bend is downward, this component is the oxidized form of the particular system represented by the curve; if upward, it belongs to the reduced form. In this way the ionization constants of the oxidized, the intermediate, and the reduced forms can be determined.

Each step of ionization changes the slope by 0.06 volt/pH in a univalent system (E_1 or E_2); by 0.03 volts/pH in a bivalent system (E_m). Hence, in the E_1 or E_2 curve there never occurs a bend such as would change the slope otherwise than by 0.06 volts/pH; and in the E_m curve there can never occur a bend such as would change the slope otherwise than by 0.03 volts/pH for each single ionization constant. These restrictions of the possible slopes facilitate the construction of such diagrams. They are, one might say, the "selection rules", the stringent necessity of which leads the way in extrapolating and interpolating over such pH stretches as might be with difficulty accessible for experimentation; for instance, it helps in ex-
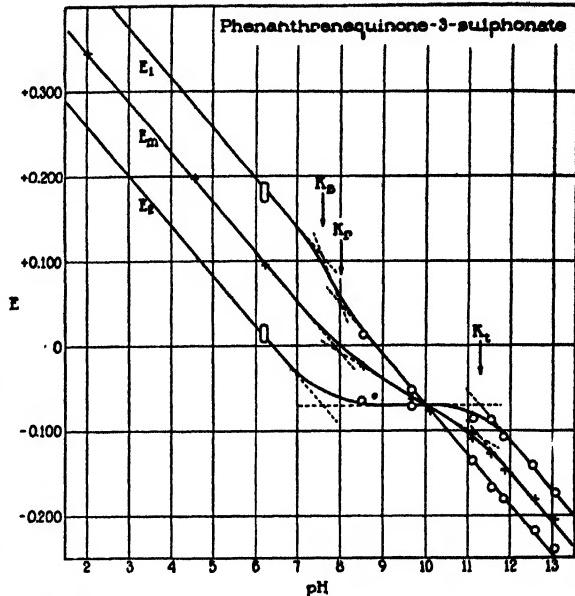
Fig. 3. Example of an anionic semiquinone. The "natural" order of the three normal potentials is on the right side from the point of intersection; the reversed order, on the left side. The three normal potentials of phenanthrene-quinone-3-sulfonate are plotted against pH. Designations as in Fig. 2. Note, in contrast to Fig. 2, the E values to the left of the crossing point are $E_1 > E_m > E_2$; to the right, $E_1 < E_m < E_2$.

trapolation to extremely high pH values where the substance may be no longer stable enough to allow a precise establishment of potentials.

We now consider the case in which the shape of a titration at a given pH depends on the concentration of the substance. The change of the shape with change of the concentration is always such that the slope at the midpoint becomes flatter as the concentration decreases. In some cases, the slope may become so flat as to coincide with a
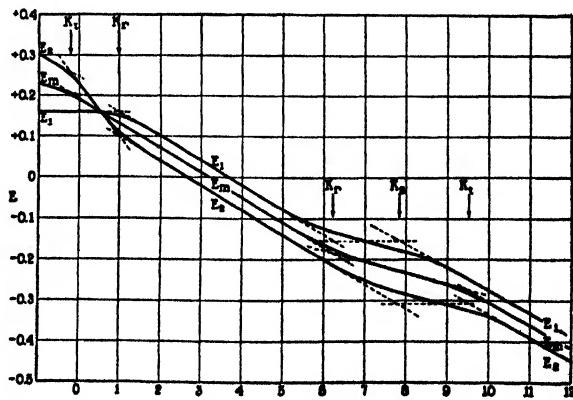
Fig. 4. The three normal potentials of lactoflavin, plotted against pH. For all pH's > 0.5, there is the reversed order of the three normal potentials.

curve with no intermediate form. An example is phenanthrene quinone-sulfonate in acid solution. In such a case no measurable amount of radical is formed, $k = 0$ within the limits of error as discussed before, and the distortion of the curve by increasing the concentration is solely due to the formation of a polymerized, and most likely a dimerized, form of the radical. Such a dimerized radical may be just as well considered as a molecular compound of the oxidized and the reduced forms. We may call it the dimeric intermediate

form, D. It is in equilibrium with the other forms in two ways

(1) $$R + T \rightleftharpoons D$$
(2) $$2\,S \rightleftharpoons D\ .$$
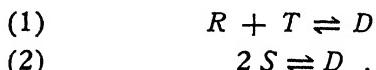
The establishment of these equilibria may not always take place with the same rapidity as the equilibrium of dismutation.

The two constants of these equilibria are, of course, interdependent. If the semiquinone forma-
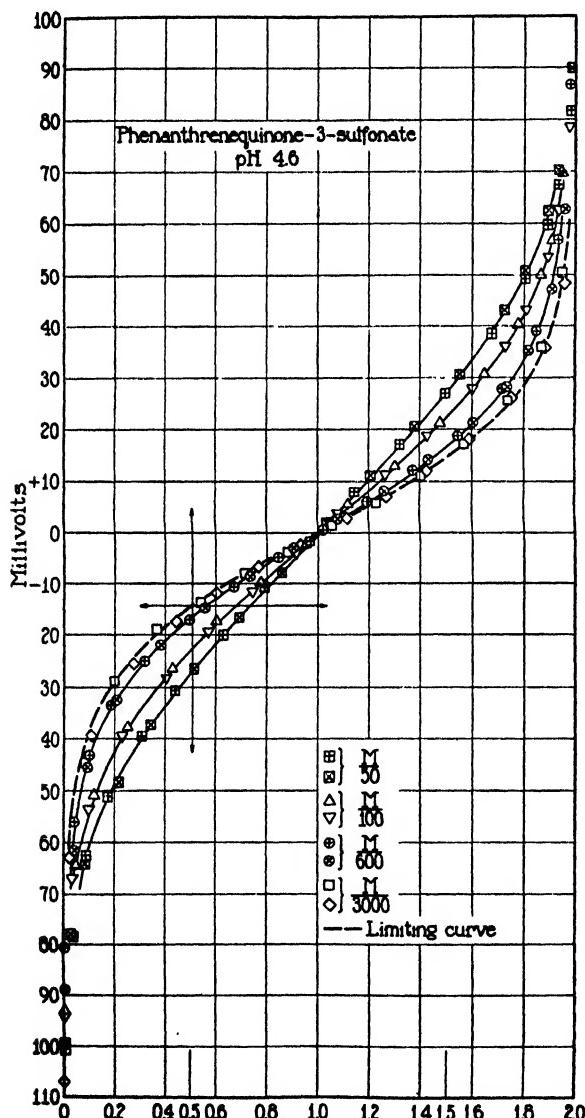
Fig. 5. Variation of the shape of the titration curve with change of concentration, for phenanthrene-quinone sulfonate at pH 4.6. The "limiting curve" is the one calculated for a bivalent system without intermediate step. The curve for the lowest concentration coincides with the limiting curve.
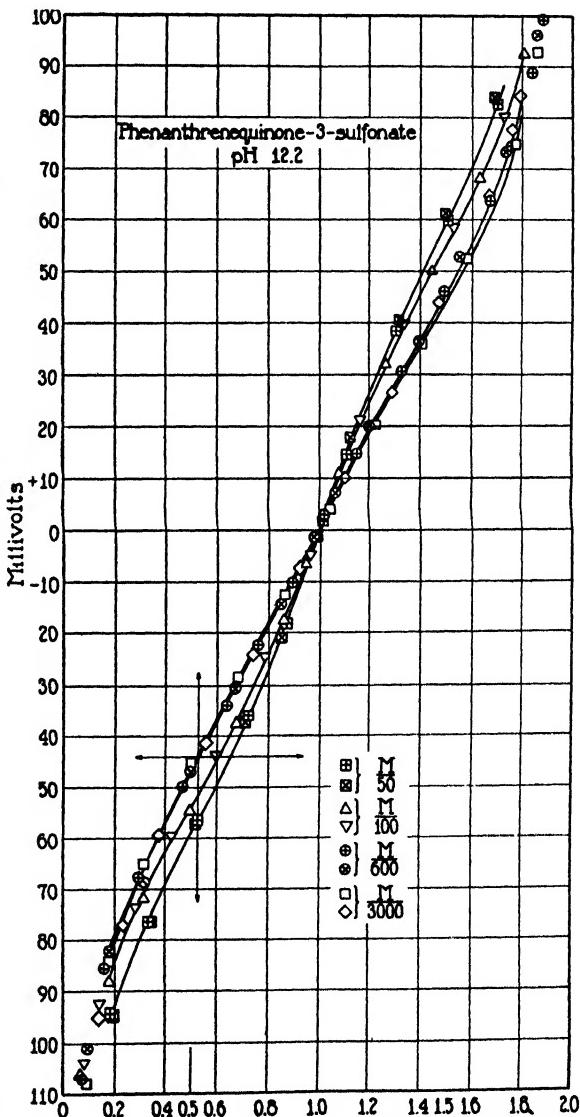
Fig. 6. Variation of the shape of the titration curve with change of concentration, for phenanthrene-quinone sulfonate, at pH 12.2. Here at the lowest concentration a limiting curve is obtained which is much steeper than a bivalent titration curve without intermediate. This is evidence for a free radical, which for higher concentrations is in equilibrium with a dimeric compound.

tion constant is zero, only the constant of reaction (1) can be determined. In other cases the effect of dilution may be such that on decreasing the concentration the slope asymptotically approaches a shape with a definite value of $E_i > 14.3$. Then it is evident that the substance is able to produce both a radical and a dimeric, and one can calculate the equilibrium constants both for reaction (1) and for reaction (2).

The method of computing these constants will be only briefly sketched here. At each point of titration, the ratio $[T] : [R]$ can be derived from the potential

$$E - E_m = 0.03 \log \frac{[T]}{[R]}$$

Furthermore, R, S, and T are correlated by the equation:

$$\frac{[S]^2}{[R][T]} = k$$

The semiquinone formation constant, k, can be derived from the asymptotic form of the curve at the lowest concentrations. Furthermore, the total concentration of the substance, a, always is

$$a = [R] + [T] + [S] + 2[D]$$

Furthermore

$$S + 2D + 2T = \text{equivalents of}$$
$$\text{oxidizing agent added.}$$

So we have four equations which can be solved for $[R]$, $[S]$, $[T]$, $[D]$. From these we calculate the dimeric formation constant g according to the equation.

$$g = \frac{[D]}{[R][T]}$$

or the dimerization constant p

$$p = \frac{[D]}{[S]^2}$$

This calculation is repeated for several points of the curve. If g (or p) turns out to be the same throughout the curve, this is evidence that the suppositions underlying the calculation are correct.

Such calculations have been performed in all details for two dye-stuffs. One is phenanthrene quinone-sulfonate. The result was this: In alkaline solution a radical is formed which is in equilibrium with a dimeric. It is possible to reach a concentration range so low that no measurable amount of the dimeric is formed, and all

the intermediate form is practically present in the form of the radical. In higher concentration the dimerization becomes more and more distinct. In acid solution no distinctly measurable amount of radical is formed. In sufficiently high concentration an intermediate form appears, which is entirely the dimeric form. These results have been confirmed by magnetic measurements, as will be shown later.

Another example is lactoflavin. Here in sufficiently dilute solution only the radical is formed, and in more concentrated solution it dimerizes in part. Fig. 7 shows how much radical, and how much dimeric is formed with varied concentrations, at pH = 6.
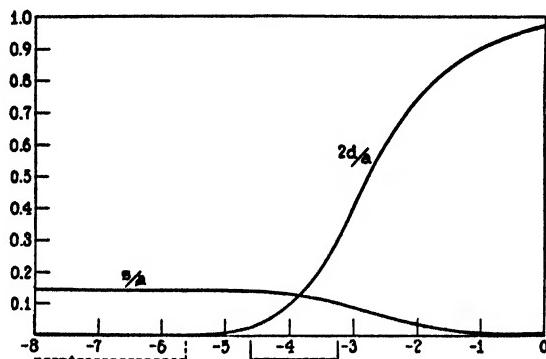
Fig. 7. The maximum ratio of semiquinone radical to total dye, $[s]/[a]_{max.}$; and the maximum ratio of the dimerized radical to total dye, $2[d]/[a]_{max.}$, plotted against log of total concentration of the dye; for lactoflavin, at pH 6. For the sake of demonstration the plot of the concentration range is by far greater than that experimentally obtainable. The bracket around —4 at the abscissa is the concentration range covered by experiments. The dotted bracket is the concentration range that may occur physiologically. It may be seen that in this concentration range there is no finite amount of dimeric compound but about 15 p.c. of the radical.

Several favorable properties of a substance must be exhibited to make possible such determinations. The range of concentration in which the variation of the form of the titration curve becomes manifest must be such that it is not necessary to consider extremely low concentrations, so low as to prevent establishment of definite potentials, and it must not be too high, because with high concentrations it is difficult to avoid change of pH and considerable changes in ionic strength during the titration. For this reason, evaluation of dimerization constants is much more difficult than that of semiquinone formation constants. The occurrence of dimerizations at those concentration ranges which are usually chosen for titration experiments is quite rare. Only recently have the two cases mentioned been discovered. When the intermediate forms are considered as they exist in

the solid crystalline state, the situation is different. Here polymerization is much more common. The significance of polymerization in the solid state, however, will not be dealt with in this paper.

2. Magnetometric Methods

G. N. Lewis first pointed out that all molecules containing an odd number of electrons should be paramagnetic, and those with an even number of electrons usually diamagnetic. The theory of magnetic properties of molecules has been greatly advanced since. The advent of quantum mechanics is responsible for an elaborate theory for the magnetic properties and has turned out to be a very complicated matter. It is, however, a fortunate property of polyatomic organic molecules containing only such elements as CHON, that the theory is very simple for such compounds. The molar paramagnetic moment of any such compound has been shown, especially by van Vleck, to be

$$\chi_{mol.} = \frac{4N \beta^2 S(S+1)}{3 K T} + N a$$

where a is the term for diamagnetism which for paramagnetic substances is practically negligible in comparison with the first paramagnetic term. N is Avogadro's number, β is Bohr's magneton, the now customary unit of magnetic moment; K is Boltzmann's constant, T the absolute temperature. S is the spin quantum number of the odd electron, which is always $= \frac{1}{2}$ for a single unpaired electron. A few organic radicals in the solid state of a type different from the semiquinones, have been investigated especially by Müller and Katz[3] and the validity of the formula has been confirmed whenever there was some evidence that the solid has the structure of a free, not polymerized radical. On the other hand, the deviation of the measurements from the expected values gives reasonable evidence as to how much the radical is polymerized.

The theory was, therefore, sufficiently elaborated as to apply to our system in which we have the radical in equilibrium with other substances in solution. However, the difficulty in carrying out magnetic measurements in such solutions seemed insuperable until recently, for the following reasons. In our dilute solution the prevailing molecular species is water, which is diamagnetic. Though its molar magnetic moment is only of the order of a few per cent (of opposite sign) of that of a free radical, the influence of the radical in a dilute solution can only amount to a slight de-

crease of the diamagnetism. This would be no disadvantage, provided we were able to compare the diamagnetism of pure water, or a suitable buffer solution, with the same buffer containing nothing but a known amount of the radical. However, we can not make up such a solution. Disregarding the fact that the radical, even when in the form of a crystalline preparation, is easily oxidized when exposed to the air in solution, still there is the difficulty that in solution the radical instantaneously dismutes and establishes equilibrium with its reduced and its oxidized, and possibly its dimeric forms. This difficulty was overcome by the following procedure. The substance is dissolved in its oxidized, quinonoid form and a slow reducing agent is added so as to bring about the reduction over a period of an hour or so, and the change of magnetic susceptibility is measured during this period. The main problem is to find a reducing agent of the desired property. Two reagents were found to be suitable. When working at pH from 11 to 13, glucose is a reductant which acts sufficiently slowly. When working at pH from 6 to 3, methylglyoxal with a suitable amount of KCN as catalyst is a suitable, slow reductant. The rate of reduction can be adapted by a proper choice of concentration. Purely qualitatively, this method could be successfully used for many dye-stuffs such as had been previously shown to form intermediate radicals by the potentiometric method. This was first shown for phenanthrene-quinone sulfonate in alkaline solution. During the period of reduction a paramagnetic substance is formed, and later disappears. In agreement with the potentiometric results, the same dye-stuff showed no paramagnetic increment during the reduction when dissolved in an acid medium (pH $= 4.6$), although an intermediate step of oxidation was clearly indicated by the appearance of a deep-brown substance during the reduction. This is a non-paramagnetic dimeric compound. In order to test the theory quantitatively one has to select some substance which forms a radical to a great extent, but no dimeric, which might complicate the calculations. Duroquinone was expected to have the desired properties according to its behavior in the potentiometric test. In fact, the agreement of the two methods was even quantitative in this case. For instance, in solution at pH 13.0, the maximum paramagnetic increment obtained during the reduction was such as to indicate according to the above formula the presence of a free radical in the fraction 0.519 $\pm$ some units of the last digit of the total amount of duroquinone present. From potentiometric experiments, at the same pH, $(s/a)_{max.}$ was calculated to be 0.517 $\pm$ 0.01. The agreement is satisfactory. The conclusion drawn from the

[3] E. Müller, I Müller-Rodloff, and W. Bunge, Liebig's Ann. *520*, 235 (1935); H. Katz, Z. Physik *87*, 238 (1933).
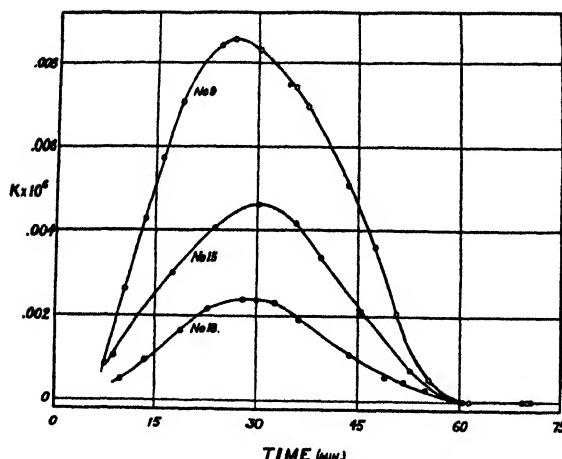
Fig. 8. Magnetic susceptibility plotted against time (minutes). Zero point of susceptibility is arbitrarily chosen, namely the time-independent value after completion of reduction. Duroquinone, at pH 13, is being slowly reduced by glucose. The three curves refer to three experiments with different initial concentrations of duroquinone.

potentiometric experiment that there is an intermediate radical and its maximum amount at pH 13 amounts to 52 p.c. of the total substance, is confirmed by magnetic measurements. This result was independent of the total concentration of the quinone used. This fact indicates that no dimeric compound is formed, in agreement with the result of potentiometry. The applicability of this beautiful method is unfortunately limited by the fact that the sensitivity of the method is sufficient only when one works in rather high concentrations. It can be used only for substances of sufficient solubility and stability. There is little hope of applying the method to substances with such a slight solubility and such a low semiquinone formation constant as lactoflavin.

3. Colorimetric and Spectroscopic Methods.

The appearance of the radical is as a rule detectable by its color, and usually even the free radical can be distinguished from its dimeric compound by its color. These are purely qualitative observations of color, which, however, can give important information, with difficulty, or not at all, attainable in any other way. The following examples show the usefulness of simple qualitative observations of color.

Distinction of radical and dimer.

When a 0.01 M aqueous solution of *p*-phenylene diamine at pH about 4 to 5, is partially oxidized with a little bromine, the first reaction is the development of a yellow color with two sharp absorption bands in the blue. This is the radical. It is not very stable in an aqueous solution but

gives rise to irreversible reactions, which are due not so much to the lability of the radical itself as to the lability of the fully oxidized form, quinonediimine. When, however, a much more concentrated solution at 0° C. of the substance is partially oxidized, the color is blue. It turns yellow on dilution. The blue compound is the dimeric form. It can be easily prepared in the crystalline state. The yellow compound arises from it by dilution and is the free radical. The experiment is even easier to demonstrate with diamino durene, where the radical is much more stable.

The polymerized form of radicals derived from aromatic diamines were first observed by Piccard, who designated them as the β forms of Wurster's salts, whereas the free radicals were called the α forms.

A similar colorimetric test for a free radical is what we may call the dilution test. It has been best elaborated for the following case. When benzil is reduced one obtains benzoin

$$C_6H_5 \cdot CO \cdot CO \cdot C_6H_5 \qquad C_6H_5CO \cdot CHOH \cdot C_6H_5$$
$$\text{benzil} \qquad\qquad \text{benzoin} \quad .$$
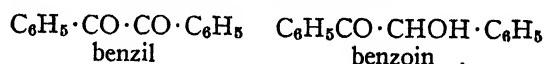
When this reduction is performed in a strongly alkaline solution, an intermediate step of reduction arises in the form of an intensely purple colored substance. If this substance be a free radical, the equilibrium would be controlled by a constant

$$\frac{[\text{benzoin}] \cdot [\text{benzil}]}{[\text{radical}]^2} = k \ .$$

If parentheses signify molar amounts and v the volume, this formula may be written

$$\frac{\dfrac{(\text{benzoin})}{v} \cdot \dfrac{(\text{benzil})}{v}}{\dfrac{(\text{radical})^2}{v^2}} = k \ .$$

So the volume cancels out; in other words the amount of radical is independent of the volume, other conditions being kept constant. If, however, the intermediate compound be a dimeric compound, one would obtain

$$\frac{\dfrac{(\text{benzoin})}{v} \cdot \dfrac{(\text{benzil})}{v}}{\dfrac{(\text{dimeric})}{v}} = k'$$

and the volume does not cancel out.

The total amount of the colored substance can be easily estimated, at least in an arbitrary scale, colorimetrically. It has been found that this amount is in fact independent of the volume. So the intermediate compound is a free radical. It exists to a measurable degree only in alkaline solution, in which it has not the composition (A), but has lost a proton (B):

$$C_6H_5 \cdot CO \cdot COH \cdot C_6H_5 \qquad C_6H_5 \cdot CO \cdot CO^- \cdot C_6H_5$$
$$(A) \qquad\qquad (B) \ .$$
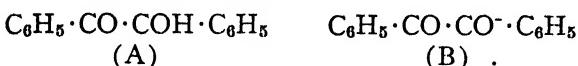
We shall see later on why the detachment of the proton as occurring in alkaline solution favors the existence of the free radical.

4. Factors Determining the Stability of Free Radicals.

The establishment of steady potentials in systems involving free radicals shows that these radicals are stable compounds, unlike such radicals as CH_3, which have been shown to exist indeed, but to have a very short life-time. The stability of such radicals even in aqueous solutions was so unexpected by the organic chemists of the classical school that it took years to overcome their reluctance to accept the existence of the semiquinone radicals. Our problem, then, is to account for the previously unexpected stability of such compounds.

The stability of a radical is not a precisely definable concept. We may define it either by the equilibrium constant of the dismutation, taking the semiquinone formation constant as a measure of the stability of the radical; or we may take the reciprocal of the constant of association to a dimeric compound as a measure of the stability of the radical. Furthermore, the radicals may have a tendency to undergo irreversible reactions when in contact with substances with which they can react, and in some cases they react irreversibly even with the solvent, especially with water, undergoing either hydrolytic decomposition or irreversible complicated dismutations, or even condensation of various kinds such as to form new dye-stuffs. This is especially true for Wurster's dye-stuffs. In such a case we may take the rate of such irreversible reactions as a scale for stability. This is especially useful when we wish to compare with each other the stabilities of the representatives of a homologous series of radicals differing only by slight substitutions. Whatever criterion one may apply, it is always apparent that the semiquinone radicals are much more stable than their structural formulae, according to classical organic chemistry, would suggest, considering the unsaturated state of such compounds, showing what may be called a valence gap.

The general answer to the question as to what circumstances are responsible for that unsuspected stability of certain radicals is that there must be a strong symmetric resonance in a free radical in order that it be a stable compound. Only such radicals as have such a resonating structure show a conspicuous stability. It should be emphasized, however, that the particular type of resonance presently to be described should not be thought of as an absolutely necessary requisite for the existence of a radical but rather as a factor greatly increasing its stability. Anyhow, all of the radicals capable of existence show this resonance to an easily measurable extent.

The concept of resonance has been developed from quantum mechanical considerations. Heisenberg used this term for the following case. Suppose an atom contains two electrons differing in their quantum numbers. Then it is impossible to state that electron 1 is in the state 1; and electron 2 is in the state 2. There is something like a permanent exchange resembling the energy exchange in two coupled pendula which causes the phenomenon of resonance. The analogy of quantum-mechanical resonance to mechanical resonance is, however, only a superficial one. What is meant by resonance is this. The real state of the two electrons can be expressed, indeed, by using quantum mechanical functions, but not by simple concepts of the kind used in classical physics. If one tries to vizualize the situation as well as can be done, one may say that the true state is, as it were, composed of the various limiting states, each contributing its share to the true intermediate state. The essential result of quantum mechanics is that the energy content of the intermediate or resonating state is lower than that of any one of the limiting states of which the resonating state is supposed to be composed. Hence, resonance increases the stability. This whole picture is something artificial, since the limiting structures to which we have to refer do not exist as chemical individuals. The practical side of this problem may be expressed as follows: if the structure of a chemical compound can be written in various ways by a varied distribution of the electrons (or the valence strokes which stand for electron pairs), without shifting any atomic nucleus, neither of these various structures is the adequate expression of the true state, rather there is resonance among all the limiting states, each contributing its share, and in consequence of this resonance the stability of the molecule is greater than any of the limiting states can account for, e.g., benzene is much more stable than any one of its imaginable formulae might suggest, due to resonance among all the possible structural formulae. If the structure of a resonating molecule is so symmetrical that two of its limiting states are undistinguishable from

each other (except for orientation in space), the increase of stability is especially large. Due to symmetrical resonance, the stability of a radical may be so much increased as to make it a compound comparable in stability with valence-saturated compounds.

All semiquinone radicals capable of existence to an easily measurable extent, or having a radical formation constant great enough to be easily measured, show this symmetrical resonance. This will be shown in a few examples.
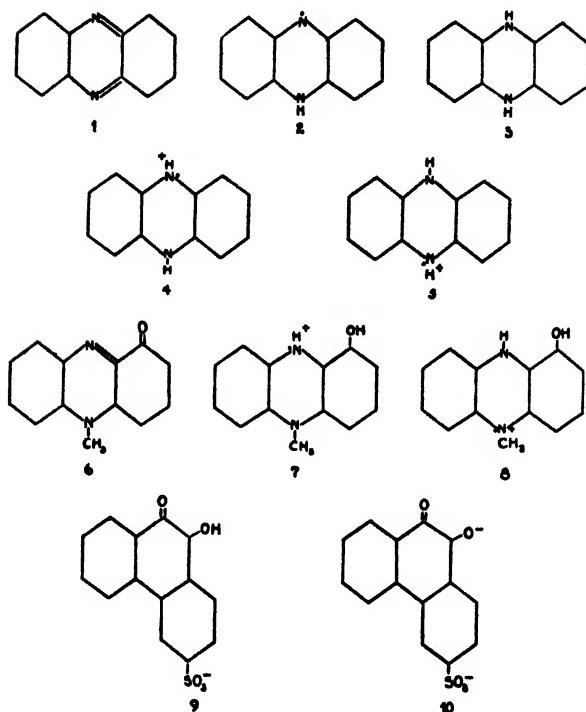
Formula (1) is phenazin, and (3) is its ordinary reduction product, dihydrophenazin. (2) represents the intermediate radical, the dot at the upper N-atom is a single, unpaired electron. If the reduction is performed in an acid solution, one proton is added. Whether this will be added to the upper or the lower N atom of formula (2), depends on which position would make the molecule more stable. If the proton is added to the upper N, a molecule with symmetrical resonance is established. So formula (2), in an acid solution, has the structure (4). This is, however, only one limiting structure of a resonance system. The other limiting structure is (5), which is undistinguishable from (4), except for orientation in space.

But even when the two limiting states are not perfectly identical there may be a strong resonance, as can be shown for the case of pyocyanine (6). When this is partially reduced by accepting

one H atom and the solution is acid enough to allow the upper N atom to accept a proton (7), this structure is in resonance with (8). (7) and (8) are not alike. Now resonance consists in exchanging electrons, and this exchange takes place from the one N atom through the conjugated double bonds of the ring to the other N atom. Within this range structures (7) and (8) are quite symmetrical, allowing for strong resonance. The essential feature here is the symmetrical location of the two N atoms. In other cases there may be two symmetrically located O atoms. The radical of phenanthrene quinone sulfonate (9) when established in an alkaline solution detaches a proton (10). Here it is undecided whether the negative charge is at the one or the other O atom, and sufficient resonance is established to make this radical stable only, however, in alkaline solution. On the other hand, the possibility that (9) becomes a more stable molecule by detaching a proton, makes the OH group of this radical more acidic than one might expect of an ordinary OH group (pK may be estimated to be 7.5 or even smaller).

Another example of symmetrical resonance is that in Wurster's dye-stuffs. These are univalent oxidation products of aromatic diamines. So, p-phenylene diamine (1), when partially oxidized, forms the radical (2) which in acid solution attaches a proton and in this form is a rather stable radical (3), due to symmetrical resonance with (4). In order that resonance be established, the
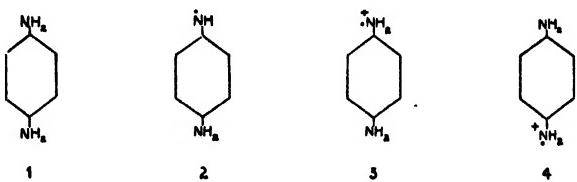
two N atoms must lie in the same plane as the benzene ring; furthermore the two atoms attached to each N (in this case H atoms) must also lie in the same plane. The reason for this requirement can be shown only by examining the local distribution of quantum-mechanical functions and is difficult to explain in terms other than quantum-mechanical ones. The validity of this requirement can be shown in the following way. When in (1) all the H atoms attached to the N atoms are substituted by CH_3 groups, the radical is formed with great ease. However, when in addition, one or more H atoms of the ring are also substituted by CH_3, steric hindrance prevents the C atoms of the methyl groups at the N from lying in the plane of the benzene ring, resonance is diminished or even abolished, and the radical becomes unstable. If one or two CH_3 groups in the ring are in *ortho*

position of a methylated amino group, the radical becomes a very labile compound; if all H atoms of the ring are methylated, no radical is formed at all. However, if the two amino groups in (1) are not substituted, the presence even of 4 methyl groups in the ring does not prevent the establishment of a stable radical.

Significance of Radicals for the Kinetics of Oxidation-Reduction.

Semiquinone radicals in reversible oxidation-reduction systems have been discovered in an ever increasing number of cases, so frequently indeed that their existence may be said to be characteristic of reversible systems. In such systems where there has been as yet no cogent evidence for their existence it can be only a matter of quantity. We have to recall that the methods of their discovery are not very sensitive. We may assume that existence and relatively high stability of these radicals is just that property which renders an oxidation-reduction system a reversible one. Irreversible systems which do not establish a definite oxidation-reduction potential in the thermodynamical sense, but may be in the best case characterized by what Conant and Fieser describe as an apparent oxidation potential, and an apparent reduction potential, are systems which do not establish an intermediate radical to any measurable extent. To understand the difference in behavior of a truly reversible system and an irreversible one, we have to consider the mechanism of oxidation or reduction when any oxidizable substance is mixed with an oxidizing agent. We propound the hypothesis that any oxidation (or reduction) has to proceed in successive univalent steps. Then, the first reaction when the oxidizing agent comes into contact with the substrate is the formation of the radical to the extent determined by the radical formation constant k. All reactions are dependent, as far as the rate is concerned, on the concentrations of the molecular species involved in the reactions. One of the factors determining the rate of the (bivalent) oxidation or reduction is the concentration of the radical. If k be very small and hence the concentration of the radical be very small, it may happen that the concentration of the radical is the limiting factor of the rate of the reaction. In such a case the oxidation of an oxidizable substance, even if the potential range of the oxidizing agent is sufficient for the over-all process, will be slow. In order to overcome the sluggishness the potential range of the oxidizing agent must be positive enough to establish the radical; the potential range of the oxidizing agent has to be not only higher than E_m, but even higher than E_1; and on reducing the oxidized form, the potential range of the reducing

agent has to be not only more negative than E_m, but even more negative than E_2. The potential range between E_1 and E_2 is the difference between the apparent oxidation potential and the apparent reduction potential.

In order that the concentration of the radical be the limiting factor for the rate of reaction, the radical formation constant must be extremely small. Such formation constants as are accessible to a measurement according to the methods described before are never small enough. So, it makes no difference for the reversible behavior of a system whether this constant is, say, 10, or 1, or even 10^{-3}. But it would make a difference if it were something like 10^{-10}. In such a case, E_1 would be about 0.3 volt more positive and E_2 about 0.3 volt more negative than the mean normal potential, and the apparent oxidation potential and the apparent reduction potential would differ from each other by something like 0.6 volt, and then the system is no longer reversible.

The function of catalysts and enzymes for oxidation-reduction would be to enter into a chemical combination with the substrate, of such a structure as to increase the semiquinone formation constant, i.e. to increase what we may call the stability of the radical, say by allowing a little more resonance than that obtaining in the radical derived from the original substrate. The mechanism by which a catalyst overcomes the sluggishness may be different according to the nature of the catalyst. As regards the physiologically occurring catalysts, we can only speculate for the time being. Such a speculation may be as follows. Suppose the radical formation constant of a substrate is too small to make it accessible to oxidation at a finite rate, the catalyst or enzyme may be imagined to be able to adsorb, or combine with, two molecules of the substrate, located in space, due to an adequate steric structure of the enzyme, in such a way as to produce a symmetry within the substrate-enzyme compound and so to enhance the resonance of the radical. If such a situation could be created by the enzyme, it would be all that is necessary to increase the rate of oxidation or reduction. There is one experimental observation by Haas which seems to indicate, in fact, that the combination of a substrate with its specific enzyme increases its ability to form a radical. When lactoflavin is used as a substrate and reduced in approximately neutral solutions by triphosphopyridin-nucleotide, an intermediate red color is observed which, according to all evidence available, is the same radical of lactoflavin which can be obtained, without enzyme, only in extremely acid solution. Whether this interpretation is correct requires further research. Since no other explanation as to the function of an oxidative catalyst has

been proposed as yet, the one suggested here will at least serve as a guide for further experimental work.

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A paper on Wurster's dyes by L. Michaelis, M. P. Schubert, and S. Granick is in *J. Am. Chem. Soc.*, 61, 1981 (1939).

DISCUSSION

Dr. Gorin: The theoretical implications of Michaelis' work on stepwise oxidation of organic compounds are of as great importance as the biological applications. There is evidence accumulating that a great many inorganic oxidation reactions are also stepwise in character. In fact, there is no definite evidence that two electrons are ever exchanged in a single step. It should be pointed out, however, that at present there is no way of definitely establishing the existence of a two electron exchange reaction. The methods employed by Michaelis, as he points out, can clearly demonstrate the intermediate only when it exists in fairly high concentrations. In no case can it be shown that no intermediate is formed. No other methods that are at present available, including those based on the study of kinetics of the reaction can, as far as I know, definitely establish the existence of a two electron exchange reaction. Without definitely ruling out the possibility of two electron exchange, therefore, it is still safe to predict that as evidence accumulates the number of oxidation reactions which are definitely step-wise in character will greatly exceed those whose status cannot be definitely established. The kinetic method, in certain types of reactions, is probably a more sensitive indication of step-wise oxidation and reduction than the more unequivocal methods employed by Michaelis.

A simple example of step-wise oxidation in organic systems is the oxidation of stannous ion to stannic tin by ferric iron. It is interesting that the reaction is extremely slow. Also the reaction is definitely bimolecular in character, strongly suggesting the formation of trivalent tin as an intermediate.

This system might also serve to emphasize another type of phenomenon. The extremely slow rate is possibly not to be explained by the high free energy of the intermediate trivalent tin. Rather it is suggested that the first step involves a rather high activation energy that arises from the electrical work necessary to bring the two positively charged ions, Fe^{+++} and Sn^{++}, close enough to each other to allow electron exchange. This effect is demonstrated by the fact that complex ions of Sn^{++} and Fe^{+++} react much more rapidly than do the ions themselves. For instance, in solutions of the chlorides of ferric and stannic the rate is at least a million times faster than with the perchlorates. Here the reacting species can be shown to be $SnCl_3^-$ and $FeCl^{++}$, rather than Fe^{+++} and Sn^{++}, a positive and negative ion rather than two positive ions. Even with the perchlorate the reaction is not directly between the ions but rather, since the reaction is greatly retarded by hydrogen ion, between hydrolysis products of iron such as $Fe(OH)_2^+$, and Sn^{++}. The smaller charge on the $Fe(OH)_2^+$ serves to reduce the activation energy arising from the repulsive forces between the positively charged ions.

Dr. Schmelkes: I would like to contribute to Michaelis' statement about formation of intermediates in simple organic oxidations. It is sometimes possible to get evidence for formation of intermediate radicals in a simple organic oxidation. For instance, if toluene is oxidized with a mild oxidizing agent such as sodium persulfate in acid solution (Wolffenstein and Moritz, Ber. Chem. Ges., *32, 25*, 1899) by vigorously stirring a benzene solution of toluene with an aqueous solution of the oxidizing agent, it is always possible to isolate a not inappreciable quantity of dibenzyl, as well as ditolyl, dimers of radicals arising from toluene by loss of one hydrogen atom. There is considerable water present in such a mixture. Even with much more water-soluble substrates, such as acetic acid, formation of succinic acid, the dimer of a radical, has been observed (Wolffenstein and Moritz, Ber. Chem. Ges., *32, 25*, 1899).

I would like to give another example where it is strikingly shown how much more stable is a resonating molecule than a molecule of similar structure which does not resonate. If, for instance, 2, 3, phenylene diamine is acted upon with HOCl an N-chloro compound is obtained which is extremely unstable. In general, those aromatic amines which do not show resonance form N-chloro compounds which are more or less unstable. One reaction that has been frequently studied is the chlorination of acetanilid. Here an unstable N-chloro compound is formed which then rearranges to a derivative containing nuclear chlorine, 4-chloro-acetanilid. But if *p*-phenylene diamine

is chlorinated an N-chloro compound is formed (Willstätter and Mayer, Ber. Chem. Ges., *47*, 1494, 1904) which derives from the quinoid form, 1-4-quinone diimine, the derivative mentioned by Michaelis as the basis of Wurster's dyes, which due to resonance is of much greater stability.
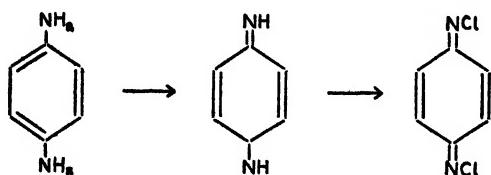
This holds true even more strikingly with other chloramines of more complex structure. We have made an N-chloro compound, N-N'-dichloro azodicarbonamidine, which shows possibilities of resonance even more than Willstätter's diimine, and the chlorine compound derived from it is even more stable. The unchlorinated azo base is a dye and shows two points of resonance. It is a dye even though it does not contain a quinoid structure.
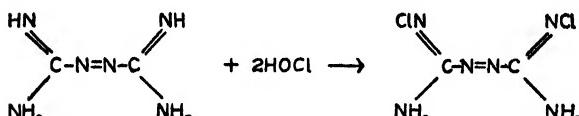
(J. Am. Chem. Soc., *56*, 1610, 1934).

Dr. Michaelis: Another beautiful example of the principle that strong symmetrical resonance favors the formation of a radical is this. Benzyl (I) can be oxidized to form benzoin (II). This is a bivalent oxidation. The problem is whether there exists an intermediate radical (III), with what may be called a tervalent C atom. When the oxidation is performed in strongly alkaline solution an intense purple color arises as an intermediate step of the oxidation. By showing that the amount of colored substance is independent of the volume of the solvent, Fetcher and I showed that the colored substance is a radical. In an alkaline solution one proton is detached, and we have (IV). Such a structure must be in resonance with (V). The resonance increases the stability of this radical; on the other hand, the possibility of (III) becoming a more stable compound by detachment of a proton makes it a stronger acid than might be expected otherwise. In neutral solution the stability of the radical, as shown in (III), is very much diminished. The radical escapes observation, not only because there is only a very small amount of it, but also because this structure exhibits no visible color. A symmetric resonance is always correlated with absorption bands in the

visible range of wavelengths, whereas lack of symmetrical resonance is unfavorable to the existence of any appreciable absorption in the visible spectrum. Time does not permit discussion of the topic of color, but strong symmetrical resonance always goes parallel with absorption in the visible spectrum. Strong resonance not only lowers the energy content of the molecule but also lowers the quantum necessary to raise its lowest energy level to the second level, thus producing absorption in the visible, instead of in the ultraviolet. The same holds for ordinary molecular species which are not radicals. The resonance, if symmetrical, always produces intense color. The type of resonance, however, is quite different in the case of ordinary organic dyestuffs. Here it is the undecidedness as to which one of two aromatic rings has a benzenoid, and which has a quinonoid structure. In a radical, the undecidedness lies in the location of the odd, unpaired electron within one ring.

$$(I) \quad C_6H_5 \cdot CO \cdot CHOH \cdot C_6H_5$$
$$(II) \quad C_6H_5 \cdot CO \cdot CO \cdot C_6H_5$$
$$(III) \quad C_6H_5 \cdot CO \cdot COH \cdot C_6H_5$$
$$(IV) \quad C_6H_5 \cdot CO \cdot CO^- \cdot C_6H_5$$
$$(V) \quad C_6H_5 \cdot CO^- \cdot CO \cdot C_6H_5$$

Dr. Barron: We have heard examples given from organic chemical reactions and inorganic chemical reactions in favor of Michaelis' theory. I, as a biologist, will give another example from biological oxidations, showing how the semi-quinone formation constant is one of the factors controlling the kinetics of biological reactions. The potential of the lactate-pyruvate system cannot be established without the interaction of an electroactive mediator. Clark has shown that this potential is better established when a mediator is chosen whose potential is the same as that of the enzymatic sluggish system. But from a kinetic point of view such a thing does not happen, because if one takes the activating proteins, lactate and pyruvate at definite ratios, and an electroactive mediator whose potential is close to that of lactate-pyruvate, *i.e.* Nile blue, the electromotive force becomes negative, and at the end of three or four hours it starts to flatten. So a definite potential is not established even at the end of eight hours. But if to such a system were added another dye which shows definite semiquinone formation, such as pyocyanine, the potential is established at the end of one hour and keeps constant. In the same manner the rate of oxidation of activated lactate is greater in the presence of pyocyanine than in the presence of methylene blue. I think these are clear examples showing how the addition of a system which shows greater semiquinone formation increases the rate of reaction.

Dr. Michaelis: May I contribute another biological example. The respiration of erythrocytes is increased by aromatic diamines. In a recent paper, Schubert, Granick and I have investigated the formation of radicals derived from aromatic diamines such as had been known as Wurster's

dyes. It was found that diamino durene easily forms such a radical when partially oxidized, but when the amino groups are methylated, no radical can be formed. This is because in the latter case there is a steric hindrance making it impossible to arrange the benzene ring, the two N atoms, and all atoms attached to each of the N atoms, in one plane. This circumstance, according to quantum mechanical considerations, must counteract resonance, and in fact it does make impossible the establishment of the corresponding Wurster's dye. Now, among all the diamines investigated, it is just this methylated diamino durene which is incapable of increasing the respiration of erythrocytes, whereas the non-methylated diamino-durene, which can form a radical, is a very active catalyst.

Dr. Wrinch: In connection with Michaelis' remarks relating to the necessity of resonance within the protein molecule, two ways in which this may occur may be pointed out. On the basis of the cyclol theory there may be resonance within the trios of OH groups on any triazine ring (Langmuir and Wrinch, Nature, *143,* 49, 1939; Langmuir, Proc. Phys. Soc. London, *51,* 234, 1939). In view of the lone pairs of electrons on the tertiary nitrogen atoms, this resonance may extend further and indeed may involve the whole cage structure. On the hydrogen-bond theory of protein structure, resonance is also possible. An example is afforded by the structures postulated by Wrinch and Jordan Lloyd (Nature, *138, 758,* 1936) where there is resonance between the amide and imide forms of the hydrogen-bonded dimerically-associated CCN skeletons, a type of structure similar to that which has been shown to exist in diketopiperazine crystals (Corey, J. Am. Chem. Soc., *60,* 1598, 1938). Evidently such resonance may also be extended further than the single dimeric unit. Each dimeric unit can be written formally in several forms (*cf.* Corey, *loc. cit.*) thus the resonance may extend over the whole closed structure formed by folding the hydrogen-bonded atomic fabric. This situation is indicated in the figure.
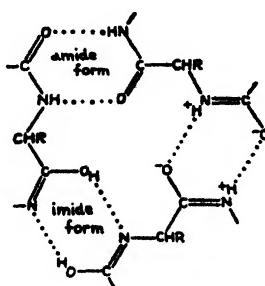
Dr. Stern: A few years ago Michaelis, in his book on oxidation-reduction potentials, put forward the general theory that enzymes are themselves reversible oxidation-reduction systems. He has now amplified this theory by further postulating that these enzymes favor the formation of semiquinones as necessary intermediates.

This brings up the question as to whether catalysts concerned in biological oxidation processes must not themselves necessarily undergo a reversible change in their state of oxidation-reduction during the catalysis. I would like to mention two cases where the experimental evidence for such a valence shift is at present insufficient.

(*1*) Knowing the ease with which flavoproteins and flavins in general may be reduced and oxidized, and that semiquinone formation has also been demonstrated in such systems, it is now generally supposed that they act only in this fashion. But there is evidence accumulating against the general applicability of this assumption. For instance, Corran and Green have isolated a flavoprotein from milk. This has the property of catalyzing the oxidation of dihydrocozymase with methylene blue. It can be shown that the flavoprotein can be reduced by chemical means and be reoxidized with molecular oxygen; still it cannot catalyze the oxidation of the coenzyme by O_2; only methylene blue may serve as acceptor. Moreover, if you bring the flavoprotein and the reduced coenzyme together, there is no reaction in the course of one hour, whereas the observed rate of the catalysis demands that such a reaction should take place in less than one second. The authors suggest, therefore, that the flavoprotein in this reaction does not undergo a cycle of the type postulated before, but that it simply catalyzes, after uniting with it, the reaction of the reduced coenzyme, with methylene blue. Dixon points out that this is a point of fundamental importance and may change many of our ideas about the role of flavoproteins in biological oxidation.

(*2*) The second example is the case of catalase. Keilin and Hartree postulate that catalase is reduced to the ferrous form by hydrogen peroxide, and that the ferrous form is reoxidized by oxygen. Theoretically, however, ferrous iron would be expected to react much more rapidly with hydrogen peroxide than with molecular oxygen. Moreover, upon exclusion of the oxygen, one would expect the ferrous form to accumulate, which, according to Keilin, has a specific absorption spectrum. However, in the absence of oxygen and in contact with the substrate, no reduction of the ferric iron of catalase has been observed as yet by spectroscopic means.

On the other hand, we have shown that if hydrogen peroxide is replaced by ethyl peroxide, an

enzyme-substrate complex is formed; there is evidence that in this complex the iron is still in a ferric form, and in no instance during the catalysis has the ferrous form been demonstrated by spectroscopic or chemical means. Theoretically, a chain reaction scheme may be formulated without involving a reduction of the ferric iron. In conclusion, I do not think that at present it is permissible to generalize by stating that all catalysts participating in biological oxidation processes must necessarily undergo an oxidation-reduction cycle during the reaction with the substrate.

THE "EQUI-VALENCE CHANGE PRINCIPLE" IN IONIC OXIDATION-REDUCTION REACTIONS

PHILIP A. SHAFFER

"The problem of reaction velocities is probably nearer to the heart of most chemists than anything else in their whole range of activity" (Tolman). In view of the undoubted concern of all chemists with these questions, it is disappointing to realize how very little is known about the factors which determine whether and how rapidly any given chemical reaction will take place. It is probably correct to say that in spite of the fund of knowledge and the rather elaborate theoretical development in the field of chemical kinetics, no principles have yet been discovered by which it is possible to predict successfully, in advance of actual test by experiment, even the approximate velocity of any dynamically possible chemical reaction.[1] From the very nature of the subject, its extreme variety and complexity, it may be too much to expect many quite diverse types of chemical change to be covered by a few generalizations. It may therefore be fruitful to look for principles applicable only to limited types of reactions. Such an effort will be outlined in the present paper.

The basic concept of all current theory concerning reaction velocity is that molecules must be in an "active" state in order that reaction may occur, and this activation is thought to consist of a minimum level of energy content and distribution, characteristic of and essential to each particular reaction. In the formulation of this basic concept of energy content only physical terms are used, no reference being made to characteristic chemical properties of the reacting species. Although this type of approach is regarded as successful in the case of certain gaseous reactions, it falls wide of the mark when applied to chemical reactions in solution. It would seem reasonable to expect that the characteristic properties of the reacting species, not at present describable by their energy content, must be given consideration in attempts to discover and understand the factors upon which depend the ability of molecules to react in specified ways. Thus, Tolman remarks at the close of his book on Statistical Mechanics, "perhaps new principles as to the behavior of individual atoms will be necessary to the final solution" of reaction velocity; and Daniels in closing his introduction to a symposium on kinetics, (Chem. Reviews *10*, 9, 1932) said, "the theory of activated molecules . . . . . seems fairly satisfactory, but it is possible that an entirely new viewpoint might be still more satisfactory". Although this need is recognized, surprisingly little progress appears yet to have been made in new directions of the sort referred to.

In a study some years ago (with Ariyama, J. Biol. Chem. *78*, LI, 1928) of sugar oxidation in alkaline buffer solutions of known pH by different oxidizing agents, we were interested to find that the rate of glucose oxidation by iodine (hypo-iodite) was much faster than by ferricyanide, when compared at the same pH and sugar concentration, with the two oxidants so adjusted as to have the same oxidation potential. The sugar activation by hydroxyl ions being identical and the oxidizing intensity of the two active oxidants being the same, we (naively) expected that the rates of oxidation might be the same. It occurred to us to wonder whether the observed difference in velocity could be ascribed to the fact that each reacting ion of ferricyanide takes one electron from the activated sugar, while each hypo-iodite ion takes two, thereby perhaps causing the reaction path and products to be different.

This thought prompted a later survey of a number of simpler inorganic oxidation-reduction reactions in a search for more light on similar problems. It was soon found that there are many cases of ionic reactions of this type which exhibit a slowness or unwillingness to react, an incompatibility between otherwise demonstrably "active" chemical substances, for which no explanation was evident. Some of the cases are very surprising and appear to be quite contrary to common-sense predictions, a situation which indicated the desirability of collecting other instances and the need to discover explanations for the strange behavior. In view of the fact that most, if not all, of these reactions were well known and many of them have been studied, it is remarkable that they have not before been assembled and their features discussed. Some of the points to be presented in this paper seem so obvious that it is difficult to believe they have not been formulated in some earlier communication. The author will be grateful if readers will inform him of earlier papers

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[1] Thermodynamics states the *possibility* of reaction under given conditions, but says nothing about probability or rate. The mass law predicts change of rate with concentration, but not the specific velocity. The Arrhenius kinetic equation calculates the velocity constant at a desired temperature, provided the velocities have been determined at other temperatures; it predicts, therefore, only an extrapolation from observed velocities.

covering the subject which he may have failed to find.[2]

Reaction Velocity in Ionic Systems. Because of the daily experience that many familiar reactions in aqueous solution between inorganic substances take place completely at ordinary temperature about as fast as the solutions are mixed (as in titrations, for example), it is often stated that for ionic reactions little, if any, energy of activation is necessary; or it is said that ions are in general in an activated state. The implications of such statements are certainly erroneous. There are many dynamically possible and definitely ionic reactions between demonstrably "active" ions which are measurably slow, while some are so slow (in the absence of catalysts) as not to be detectable. In many such cases the slow rate cannot be the result of "inactivity", in the sense of requiring energy of activation, because each participant may react very rapidly under the same conditions with different reaction partners. And in some instances it can be shown that two thermodynamically reversible and electromotively active systems, between which the free energy permits substantially complete reaction, nevertheless fail to react when mixed in the same solution. Being electromotively active, they do react at once when separated by a bridge or porous cup and joined through electrodes by a metallic circuit as the half-cells of a battery. Such cases obviously constitute the seemingly bizarre phenomena of chemical reactions that take place rapidly at a distance and indirectly (by electron transfer through electrodes), but do not occur directly in a homogeneous solution. The electrodes may be said to catalyze the reactions.

In agreement with this idea, it was found in a number of instances that the addition of certain third substances exerts a marked acceleration on these slow reactions. It therefore seemed hopeful that discovery of the cause for the slowness of these ionic reactions might supply also an explanation of the action of catalysts in these cases.

To make clearer the sort of phenomena being discussed, one of the most striking illustrations to

which attention was drawn some years ago (J. Am. Chem. Soc. *55*, 2169, 1933), will be referred to again. Ceric sulphate in about N H_2SO_4 is a powerful oxidant which reacts instantly with many reductants and has become a popular reagent in volumetric analysis. With cerous ion it forms a reversible electromotively active couple of high potential (1.44 v.) and is accordingly used also in potentiometric titrations. The electrode potentials are evidence of the "activity" of the ions. There are nevertheless a number of reductants, some of marked reducing intensity, which are not oxidized by ceric ion, or are oxidized very slowly. For example, hypophosphorous acid, H_3PO_2, reduces cupric ion to hydride and liberates H_2 gas, reduces sulphite, and behaves in other ways as a powerful reducing agent. Yet its oxidation by ceric ion is exceedingly slow at room temperature.

A much clearer case is the reaction of ceric with thallous ions. Thallous and thallic sulphates in sulphuric acid, like ceric-cerous, form a reversible electromotively active system, the potentials of which are accurately measurable by noble metal electrodes; $E_o = 1.24$ v. When an electrolytic cell is made with ceric and thallous sulphates in N H_2SO_4 as the half-cells, current flows and ceric ion is reduced while thallous ion is oxidized. The difference between the normal potentials permits reduction of more than 0.999 of the ceric ions by a half mol (one equivalent) of thallous ion; *i.e.* the reaction is nearly complete at equilibrium. When, however, the two solutions are mixed, no appreciable reaction takes place even on boiling. But if a small quantity of manganese sulphate is added to the solution, reaction then proceeds even at room temperature at rates approximately proportional to the amount of the catalyst added.

The simplest explanation of these facts seems to be the following. The valence change involved in the reduction of ceric ion Ce^{4+} to Ce^{3+} is the addition of one electron, no lower valence state of Ce being known. With thallous ion, however, the only apparent possible valence change (Tl^+ to Tl^{3+}) is the loss of two electrons; no compounds of Tl^{2+} are known and this valence state appears not to exist. Reaction between Ce^{4+} and Tl^+ would therefore seem to require a three-body collision, a tri-molecular reaction, which in this case does not occur. This idea is supported by the facts, first that Mn^{2+} is promptly oxidized to Mn^{4+} (MnO_2) by Ce^{4+}, presumably stepwise *via* Mn^{3+}; and second that Mn^{4+} promptly oxidizes Tl^+ to Tl^{3+}. The hypothesis is, therefore, that Ce^{4+} fails to react with Tl^+ at measurable speed because of the impossibility of doing so by bi-molecular reaction. Mn^{2+} acts catalytically by its ability to mediate the reaction, first by yielding

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[2] A somewhat similar idea was proposed by Kirk and Browne (J. Am. Chem. Soc. *50*, 337, 1928) from their study of the oxidation of hydrazine by different agents. They classified different oxidants as "mono- di-, and complex delectronators". Another similar hypothesis is that of Hale (J. Phys. Chem. *33*, 1633, 1929), who explained the induced oxidation of arsenite by O_2 when it oxidizes ferrous pyrophosphate in alkaline solution by supposing that the ferrous complex (containing two Fe atoms) by combining with a molecule of O_2 is oxidized to the 4+ state (Fe_2O_4) which in turn yields to arsenite one O atom. He suggests that O_2 fails to react directly with arsenite because the latter can take up only one atom of O, which O_2 refuses to part with. Both these concepts are closely related to the ideas presented here.

two electrons consecutively to two Ce^{4+} ions and then by withdrawing a pair of electrons in one step from Tl^+ ion, the potential or free energy change of each step being such as to permit its occurrence. That this is the correct explanation of this case would seem probable from the fact that Ce^{4+} is similarly sluggish in reacting with a number of other reductants which appear to yield only, or preferably, a pair of electrons. And on the other hand, Tl^+ fails to be oxidized also by at least one other sufficiently intense oxidant, vanadic acid, which can accept only one electron (at the necessary energy level).

There are several considerations which appear to support the view that the "equi-valence change principle" illustrated by the cases cited above may be of rather general applicability. The main considerations will be briefly enumerated.

1. It is almost always found that reactions having measurable rates are either first or second order processes, whatever may be the stoichiometric equivalents in the over-all reaction. A few third order reactions appear to occur, but probably none of a higher order. Kinetic theory and statistical mechanics predict that this should be the case, because the probability of simultaneous approach of three molecules is something like 10^{-6} less than for a two-body collision, while a simultaneous collision of more than three is exceedingly improbable.

2. The large body of facts represented by the grouping of the periodic table demonstrates certain preferential valence states for each element. If one makes a list of the elements in the order of their atomic numbers and puts down opposite each its known valence states, it is found that a regularity exists not only in maximum positive and maximum negative states, but also in lower or intermediate states, and therefore also in the *missing* valence states. Among the elements which exhibit plural valence states, those of the first and second short periods and those at the *ends* of the long periods show (with some omissions and exceptions) a systematic *alternation* in pattern, either 1, 3, 5, 7 or 2, 4, 6; while those which constitute the first cycles of the long periods combine both patterns and exhibit a more nearly complete series of states. The latter condition applies also (in some degree) to most of the members of group V in all periods (N,P,V, [As ?] Cb,Sb,Bi).

Viewed in another way we find like differences in behavior of the elements when arranged in valence groups.

The existence of these preferential states may be looked upon in either of two ways: that the neutral atom and $1+$ ions of the alkalies of group I, for example, are the only *stable* states (others, for example Na^{2+}, being *possible,* but of fleeting existence, because unstable) ; or that as regards chemical reactions only the known states are "possible", and that no other ion or molecule is able under ordinary circumstances to remove (for example) a second electron from Na^+ to give Na^{++} ion. The first view is that of physics in measuring ionization potentials of the elements. As regards chemical reaction, the second view seems the more probable. From the second view, we may speak of "permissible" and "impossible" valence states, and may suppose that only those valence states are "possible" which are represented by known chemical compounds.

3. Combining these ideas it may be expected that only those ionic oxidation-reduction reactions will be rapid in which (1) the ions are active or become activated, (2) their "possible" valence states permits an equal transfer of electrons from reductant to oxidant by bi-molecular collision or a sequence of these, and (3) provided, of course, a decrease of free energy permits the transfer at each step.

4. In cases where a transfer of electrons is prevented by "impossible" valence states, the incompatibility might be mediated by a third reactant able by reason of its "possible" valence states (and potentials) to accommodate both.

5. Another means of mediating an unequal valence change would be salt or complex formation resulting in the union of two ions of a monovalent reactant, thus permitting it to react in pairs as a bivalent reactant. Various forms of this type of mediation appear to occur. If a temporary complex be formed between the primary "unequal" valent-change reactants, they would in effect not be unequal, the process consisting of two stages, the first incomplete, of equi-valent reactions.

Another type appears to be salt formation illustrated by the following case. Titanous chloride in HCl solution, although an intense and rapidly acting reductant, reduces I_3^- ion only slowly; the reduction of even Cl_2 is by no means instantaneous. The rates are markedly accelerated by adding H_2SO_4, H_3PO_4, citric acid or their alkali salts, an effect which may be due to the formation of poorly dissociated titanous salts, in which two Ti^{3+} ions are held together, thereby permitting the loss of two electrons to I_2, yielding two I^-.

Testing the "Equi-valence Change Hypothesis". An adequate test of the ideas outlined above is difficult, if not at present impossible, for several reasons. Study of a few cases alone will not provide a test of so general an idea; a broad survey, even if superficial, is first necessary. Quantitative data on reaction velocity, although essential, are often difficult to use because of uncertainty as to what step or process is being measured. Furthermore, reaction velocity has

been determined only of a few moderately slow (measurable) processes. For our purpose we shall therefore have to use a crude classification of (1) seemingly very rapid reactions which go at once virtually to completion on mixing the solutions at room temperature, (2) those which are perceptibly slower, requiring a few seconds or minutes, (3) those of measurable rates with half-periods of hours and (4) those which are very slow or appear not to take place. Examples of all groups are to be found among dynamically possible ionic reactions.

We shall consider here a number of reactions found by experience to be serviceable as titrametric methods in analytical chemistry. These are, of course, generally very rapid and substantially complete reactions, and according to hypothesis should be equi-valence change processes, or a sequence of these. Conversely, there are many dynamically possible reactions which have been found too slow for direct titrametric methods, and these should in general be "unequal" reactions. Where catalysts are effective in these otherwise slow reactions, a brief analysis of their role will be attempted.

Oxidation by Ceric Ions. In strongly acid solution (to prevent hydrolysis) Ce^{4+} and Ce^{3+} constitute a reversible electromotively active couple of high potential. In H_2SO_4 solution, $E_o = 1.5$ v. Ceric sulphate is a popular analytical reagent, being used as a substitute for permanganate by both colorimetric and electrometric titrations for determination of a variety of substances. The only known valence states (except the peroxide, Ce^{6+}) are Ce^{4+} and Ce^{3+}, and the reduction corresponds to one electron equivalent per mol.

Table I lists a number of substances which are rapidly oxidized without catalysts at room temperature by ceric sulphate in N H_2SO_4 solution, and in another column certain other substances which fail to react or do so very slowly unless catalysis or high temperature or both are used. All of the reductants in the list of those rapidly oxidized, except H_2O_2 and H_2SO_3, are one-valence change substances. H_2O_2, presumably a divalent reductant, is peculiar in some other reactions, and its rapid reaction with Ce^{4+} may be related to formation of the known per-ceric compound. Hydroquinone, like a number of similar organic substances, represents a type now known to be capable of mono- or di-valent change (and is therefore catalytic in some "unequal" re-

Table I

Reactions with Ceric Sulphate.

$$Ce^{4+} + e \rightleftharpoons Ce^{3+} \qquad E_o = 1.45 \text{ v.}$$

(Reactions in about M H_2SO_4.)

Rapid at room temperature		Slow at room temperature unless catalyst is used.		
Reductant	Valence Change	Reductant	Valence Change	Catalyst
Ti^{3+}	1	Hg_2^{++}	2	Boiling. Mn^{++}
Fe^{2+}	1	Tl^+	2	Cl^-, ICl, Mn^{++}
Ferrocyanide	1	As^{3+}	2	Cl^-, I^-, ICl
Vanadyl $(VO)^{2+}$	1	Sb^{2+}	2	Cl^-, I^-, ICl
Phenanthrolene-ferrous complex	1	Cr^{3+}	3 (?)	Boiling. HNO_3
		H_2SeO_3	2	HCl, ICl
Br^-, I^-	1	H_2TeO_3	2	Cr^{3+}. Boiling
$Mn^{2+} \rightarrow Mn^{3+} \rightarrow Mn^{4+}$	1 each	H_2S	2	I^-
Hydroquinone	2 or 2 × 1	H_3PO_2	2 or 4	I^-, Br^-, Cl^-, ICl
H_2O_2	2			
H_2SO_3	2 (?)			

actions). The oxidation of H_2SO_3 is exceptional in that a large fraction of the product is di-thionic acid (Benrath, Z. anorg. Ch. *114, 267, 1920*).

In contrast with these rapid reactions, the substances listed as reacting slowly and requiring catalysts or high temperature, all demand valence changes of two or more. Reaction is made possible or greatly accelerated by the catalysts named. In nearly all cases the mechanism of the catalysis appears to be mediation of the disparity of electron capacity of oxidant and reductant, thereby permitting a sequence of bi-molecular equi-valence change reactions.

Phenanthroline-ferrous complex. This substance (Blau) and its oxidation product afford interesting cases for test. They represent a reversible electromotively active couple of high potential, now used as indicators in titration with $KMnO_4$ or ceric sulfate. (Walden, Hammet and Chapman, J. Am. Chem. Soc. *53, 3908, 1931; 55, 2469, 1933*). According to Blau's analyses the molecule contains 3 phenanthroline groups and 1 Fe, and the valence change on oxidation is merely the change from Fe^{2+} to Fe^{3+}, as shown by the titration given by Walden and associates, in which $N = 1$. They calculate E_o in M H_2SO_4 as 1.14 v. (On repeating the same titration we find E_o in M $H_2SO_4 = 1.06$ v.) It requires, therefore, powerful oxidants to bring about the oxidation, while the ferric form of the complex is a powerful oxidant. On this property rests its use as an indicator; a small excess of Ce^{4+} or MnO_4^- at once causes oxidation. (These are both one-electron oxidants.) HVO_3 reaches the necessary potential only at high acidity, and when no more than about 5 p.c. reduced, but at higher acidity the dye is oxidized by large excess of HVO_3. Tl^{3+} in M H_2SO_4 has much higher potential, but does *not* oxidize the dye. Nor do HIO_4, $HBrO_3$, nor Br_2 nor Cl_2 (!), all in M H_2SO_4, oxidize it, though with each the potential would permit the reaction to occur. In dilute acetic acid or water alone (where HOCl may form) Cl_2 causes oxidation. Oxidation also occurs rapidly on warming, by HIO_4 in the presence of Mn^{2+} (which forms MnO_4^- and MnO_2, thus permitting one-electron reaction).

The reverse is equally striking. After oxidizing the complex with a minimum amount of Ce^{4+} in M H_2SO_4, the dye is instantly reduced by V^{4+}, Fe^{2+}, I^-, $FeC_6N_6^{4-}$, hydroquinone, N_2O_2, and slowly by metallic Hg and Ag. It is *not* reduced by large excess of Tl^+, nor by $Hg_2(HO_3)_2$ (the potential of the solution used was $E_h + 0.83$ v., with H_2SO_4 0.9 v., or about 0.25 v. lower than that of the phenanthroline-ferric solution). Reduction occurs (in M H_2SO_4) rapidly with H_2SO_3, $H_2S_2O_3$, and slowly at room temperature with large excess of arsenite. The dye catalyzes

the reduction of Ce^{4+} by arsenite in M H_2SO_4. How these apparent exceptions to the "equivalence principle" can be accounted for is undecided.

Thallic and Thallous Ions. Thallous (Tl^+) and thallic (Tl^{3+}) ions in H_2SO_4 solution form a reversible electromotively active couple, normal potential $+ 1.2$ v. The intermediate valence state of Tl^{2+} probably does not exist; its compounds are unknown, and the potential titration curve gives no hint of an intermediate valence state. In the presence of Cl^- or other halide ions, due to complex formation, the potential is lowered about 300 mv. Table II lists a number of reductants and oxidants reacting with these ions. Thallic sulphate in N H_2SO_4 is rapidly reduced at room temperature by H_2SO_3, hydroquinone, Sn^{2+} and moderately rapidly by mercurous nitrate, all two-electron change reductants. It is not reduced even on boiling by vanadyl sulphate (VO^{++} or V^{4+}), although the potentials permit about half reaction with equivalent concentrations. With Fe^{2+}, reduction of Tl^{3+} is rather rapid, but is far from being instantaneous. If to a solution of $FeSO_4$ in M H_2SO_4 containing sulphocyanate there be added Tl^{3+} in H_2SO_4, the red color of ferric sulphocyanate gradually increases. The same experiment with V^{5+} or Ce^{4+} as the oxidants yields maximum color at once.

Similarly with ferrocyanide Tl^{3+} is only slowly reduced, although a precipitate forms at once. The addition of Fe^{2+} to a mixture of Tl^{3+} (excess) and $K_4Fe(CN)_6$ in N H_2SO_4, gives no reaction for ferricyanide; but on adding Fe^{3+} Prussian blue forms at once.

With titanous chloride Tl^{3+} is only slowly reduced, although both are electromotively active. Electrometric titrations are not smooth, the potentials jump several hundred mv. and change rapidly after each addition, indicating a sluggish reaction catalysed by the electrode surface. The reaction between Ti^{3+} and Tl^{3+} is strikingly catalysed by Cu^{2+}, indigo disulphonate, and by phospho- and arseno-tungstates.

Thallous sulphate is rapidly oxidized by Cl_2, Br_2 and rather rapidly by suspensions of (solid) MnO_2, $HBiO_3$ and PbO_2; also by cobaltic oxide. It is not oxidized (without catalysts) by Ce^{4+}, HVO_3, H_2CrO_4 and only slowly by $HMnO_4$. On adding and excess of $KMnO_4$ solution to thallous sulphate in N H_2SO_4, a crystalline precipitate of $TlMnO_4$ forms, which gradually decomposes, yielding MnO_2 and Tl^{3+}. The filtrate contains both Tl^+ and MnO_4^- ions.

The Oxidation of As^{3+} and Sb^{3+} with Iodine-Chloride Catalysis. The oxidation of arsenite (As^{3+} to As^{5+}) and of antimonite (Sb^{3+} to Sb^{5+}) by $KMnO_4$ in acid solution is not smooth or rapid and is, therefore, not recommended as

Table II

Reactions with Thallic Sulphate.

$$Tl^{3+} + 2e \rightleftharpoons Tl^+ \qquad E_o = 1.2 \text{ v.}$$

Rapid reactions		Slow reactions unless catalysts are used.		
Reductant	Valence Change	Reductant	Valence Change	Catalyst
H_2SO_3	2	$(VO)^{2+}$ (V^{4+})	1	Cl^-
Hydroquinone	1 or 2×1	Fe^{2+}	1	
Sn^{2+}	2	Ti^{3+}	1	Cu^{2+}, indigo disulphonate
Mercurous nitrate	2	Ferrocyanide	1	

Reactions with Thallous Sulphate.

Rapid Reactions		Slow Reactions		
Oxidant	Valence Change	Oxidant	Valence Change	Catalyst
Cl_2 or Br_2	2	Ce^{4+}	1	HCl, Mn^{2+}
MnO_2	2	$HMnO_4$		
$HBiO_3$ } solids	2	H_2CrO_4		HCl, Br^-
PbO_2	2	HVO_3	1	

an analytical process even at boiling temperature. This is to be expected since the valence change of these reductants is 2, a change impossible to MnO_4^-. The positive valence states of Mn are 2+, 3+, 4+, 6+, 7+ (no 5+). With ceric sulfate (Ce^{4+} to Ce^{3+}) and HVO_3 the oxidation of As^{3+} or Sb^{3+} is not rapid, presumably for the same reason. As^{3+} and Sb^{3+} are, however, rapidly oxidized by various oxidants capable of a 2 valence change; namely, by I_2 (or HIO) in bicarbonate solution, by ICl, Cl_2, Br_2, Tl^{3+}. HIO_3 with high HCl concentration in which ICl is the end product of the oxidant (Jamieson) or in the presence of HCN with which ICN is the end product (Lang) are the quantitative methods recommended (Kolthoff-Furman) for As and Sb determination. These reactions can be written as "equal" two-valent change bi-molecular reactions.

The slow "unequal" reactions of As^{3+} or Sb^{3+} with $HMnO_4$ or Ce^{4+} become smooth and rapid at room temperature when ICl or I^- or IO_3^- is added to catalyse the process, provided a sufficient concentration of H^+ and Cl^- ions be present to permit reformation of ICl from I^- or I_2 formed when ICl is reduced or when it reacts with I^-. The details of the processes need not be written out here; they become fairly obvious from an

application of the equi-valence change principle to the results of Willard, Furman, Swift, and their co-workers, whose papers as well as those of Lang, Jamieson and others are cited and discussed in the book of Kolthoff and Furman on volumetric analysis (1929), and in the second edition (1931) of "Potentiometric Titrations" by the same authors.

Iodine chloride may be formed from I^- either by two consecutive 1 valence steps, or by a single 2 valence step.

$$I^- \rightarrow I + e; \; 1/2(I_2) \rightarrow I^+ + e$$
$$I^- \rightarrow I^+ + 2e; \; I^+ + Cl^- \rightarrow ICl.$$
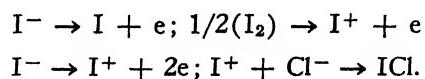
The single valence changes are readily accomplished by Ce^{4+} or by $HMnO_4$ while the two-valence change is accomplished by MnO_2 in HCl solution. (MnO_2 and $HMnO_4$ are formed in acid solution from MnO_4^{--}, left when MnO_4^- accepts 1 electron.) ICl acts then as a 2 valence oxidant:

$$ICl + 2e \rightarrow I^- + Cl^-.$$
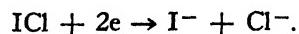
This would seem to be the explanation of the action of ICl (or I^- or Br^- or IO_3^-) catalysis in these reactions; it "accommodates" two re-

actants "unequal" toward each other (Ce^{4+} to Ce^{3+}; As^{3+} to As^{5+}) by its formation through two *one* valence changes, and by its reduction by one *two* valence change.

Titanous and Stannous Ions. A comparison of the rates of reduction of various oxidants by Ti^{3+} and Sn^{2+} ions is interesting and appears to be significant. Each reducing agent acts rapidly toward certain oxidants, but slowly or not at all toward others with which reaction is dynamically possible. The reducing intensities are not far different, the normal potential of Ti^{3+} — Ti^{4+} being 0.05 v. and that of Sn^{2+} — Sn^{4+} being 0.13 v. The valence change when Ti^{3+} is oxidized corresponds to the loss of one electron, while with Sn^{2+} two electrons are lost. The existence of Sn^{3+} state is very doubtful. Although part reaction between Ti^{3+} and Sn^{4+}, and between Sn^{2+} and Ti^{4+} is dynamically possible, no reaction occurs, a fact noted by Knecht and Hibbert.

On listing for comparison the relative rapidity of reaction by various oxidants with Sn^{2+} and Ti^{3+} in HCl or H_2SO_4 solution, one is struck with the fact that in a number of cases where reaction with Sn^{2+} is rapid it is slow with Ti^{3+}, and *vice versa*; and furthermore that the oxidants rapidly reduced by Sn^{2+} can accept two electrons, while those rapidly reduced by Ti^{3+} accept only one. This is what one would expect from the "equi-valence change principle". Table III gives a survey of some of these cases.

TABLE III

Reductions by Titanous and Stannous Ions.

(In 2N HCl or M H_2SO_4 at room temperature.)

	Ti^{3+}	Sn^{2+}
Ti^{4+}		—
Sn^{4+}	—	
Hg^{2+} (0.9 v.)	Slow	Fast
Tl^{3+} (0.9 v. in HCl)	Slow	Fast
I_2 (0.5 v.)	Slow	Fast
Br_2	Slow	Fast
As^{5+} Sb^{5+}	Slow	Faster
Fe^{3+} (0.7 v.)	Fast	Slower
Ag^+ (0.7 v. in H_2SO_4)	Fast	Slow
V^{5+}	Fast	Fast
Bi^{3+}	Fast	Fast
Indigo disulphonate and many dyes	Fast	Fast (?)

Reduction of I_2 (KI + I_2 in HCl or H_2SO_4 solution, $E_0 = 0.54$ v.) is instantaneous with Sn^{2+}, but slow with Ti^{3+}, the rate of which reaction has been measured (Yost, Zabaro, J. Am. Chem. Soc. *48*, 1181, 1926). The rate is accelerated by Cu^{2+}, indigo disulphonate, molybdic, arseno- and phospho-tungstic acids, which are reduced by Ti^{3+}, and the reduced forms are oxidized by I_2. It can be shown that each of these catalysts can accept two electrons in steps of one each, and yield two in a single step.[8] Tendency to relative slowness also may be detected in the reaction of Ti^{3+} with Br_2 and even with Cl_2 in HCl solution.

With Tl^{3+} also (a two electron oxidant of high intensity) reaction with Sn^{2+} is much more rapid than with Ti^{3+}; this is indicated by the addition of chromate to stoichiometric mixtures of the two combinations, which yields at once with the Sn^{2+} containing mixture a precipitate of thallous chromate, but not with Ti^{3+}.

Reduction of As^{5+} and of Sb^{5+} are likewise extremely slow at room temperature with Ti^{3+}, and more rapid with Sn^{2+}. The electrometric titration of Fe^{3+} by Ti^{3+} is said not to be interfered with by the presence of Sb^{5+} or As^{5+}, which is not the case with the titration of Cu^{2+} because it catalyzes the reduction of antimony.

$$2Cu^{2+} \overset{(2 \times 1 \text{ e})}{\underset{(2 \text{ e})}{\rightleftharpoons}} Cu_2^{++}$$
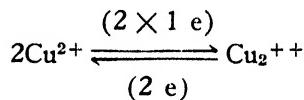
With Fe^{3+} the situation is reversed; being reduced instantly by Ti^{3+}, reaction is slow with Sn^{2+}. (Analytical titrations must be made at 75° C.) Vanadic acid is reduced rapidly by both Ti^{3+} and Sn^{2+}, presumably due to the fact that V^{5+} may be reduced by Sn^{2+} to V^{3+}, which reacts with V^{5+} to give V^{4+}.

Knecht and Hibbert ("New Reduction Methods", p. 6) mention the "curious anomaly" that "whereas the much weaker stannous chloride instantly precipitates white mercurous chloride (metal with excess Sn^{2+}) in the cold, titanous chloride is without action under these conditions, and only reacts on boiling the solution, when reduction takes place. Conversely mercury in large excess reduces titanic to titanous chloride in the cold." The potentials of $2Hg^{2+}$ — Hg_2^{++} (0.92 v.), of Hg^{2+} — Hg. (0.86 v.), and of Hg_2^{++} — 2Hg (0.62 v.) in H_2SO_4 (or even in HCl) are far above the levels of Ti^{3+} and reduction to Hg metal is dynamically possible.

[a] The reaction between Ti^{3+} and I_2 is accelerated also by the addition of di- or poly-basic acids (H_2SO_4, H_3PO_4, tartaric and citrate). This effect is thought to be due to forming complex or salts containing two or more Ti^{3+} ions.

Reduction at room temperature is accelerated with catalysts or on long heating. The explanation of the slowness or failure of these reactions with Ti^{3+} appears to be that the process is $Hg^{2+} \to Hg$ and $Hg^{2+} + Hg \rightleftharpoons Hg_2^{++}$, two electrons being involved, a number which can be supplied by Sn^{2+} but not by Ti^{3+}. With Ti^{3+}, $BiCl_3$ is found to be an effective catalyst for Hg^{2+} reduction (Zintl, Rienäcker, Z. anorg. allgem. Chem. *155*, 84, 1926). Since valence states of $1+$, $2+$, $3+$ $(4+)$ and $5+$ of Bi are known (all reduced to metal by both Sn^{2+} and Ti^{3+}) it is not surprising that Bi salts are catalysts in this case. Other catalysts of this reaction doubtless act in the same way. Indigo disulphonate, phosphotungstic and arseno-tungstic acids have catalytic effect.

In strongly acid solution Ti^{3+} reduces H_2SO_3 to $H_2S_2O_4$ (Knecht, Hibbert). Although not quite instantaneous, it is rapid at room temperature. The process probably is: $Ti^{3+} + H_2SO_3 + H^+ \to Ti^{4+} + HSO_2 + H_2O$, and $HSO_2 + HSO_2 \to H_2S_2O_4$. As would be expected, this reaction catalyses reduction of Hg^{2+} by Ti^{3+}; Hg_2SO_4 and Hg separate promptly from a mixture of Ti^{3+} and Hg^{2+} in H_2SO_4 solution when H_2SO_3 is added.

Hypophosphorous Acid. There are many curious reactions of this substance for which no explanations have been given. We shall note here only a few cases by way of illustration. On heating, H_3PO_2 decomposes into PH_3 and H_3PO_4, presumably an even 4 electron transfer between 2 activated molecules; the oxidation states are 3^- in PH_3, 1^+ in H_3PO_2 and 5^+ in H_3PO_4. On oxidation $H(H_2PO_2)$ passes (by loss of 2 electrons and addition of O^{--}) to H_3PO_3 which, by loss of a second pair (and addition of O^{--}), gives H_3PO_4; presumably $H(H_2PO_2)$ may also lose 4 electrons at a single step and pass directly to H_3PO_4, but 1 or 3 valence changes seem to be "impossible". These expectations appear to account, to some extent at least, for the behavior of these substances.

Hypophosphorous acid, although strong enough as a reducing agent to reduce Cu^{++} to Cu and to CuH_2, being converted in each case to H_3PO_3 —each of the processes being a 2 valence step— is strangely resistant to oxidation by chromic acid; even on boiling in dilute solution $K_2Cr_2O_7$ acidified by H_2SO_4 is only slowly reduced; a 2 electron change is apparently not possible for CrO_4^{--}. Catalysis is marked with I^- or Br^-.

On the other hand, H_2SO_3 and H_2SeO_3 are both promptly reduced to S and Se respectively on moderate warming (slowly at room temperature), with formation of H_3PO_3. Rather striking examples of induced reduction may be observed with H_3PO_2 and H_2SO_3 or $CuSO_4$. None of these substances reduces methylene blue, 1-5 anthra-

quinone or molybdic acid (to blue pentavalent state); but when to a solution of H_3PO_2 (about 0.1 M) containing either methylene blue, anthraquinone, molybdic acid, or phosphomolybdate, a few drops of sulfite solution are added, these acceptors are promptly reduced. Because of the color changes they make pleasing demonstrations. The reactions with methylene blue and anthraquinone, if on reduction they accept a pair of electrons, would seem to be "equal" reactions; why they fail to proceed is not evident, unless it be that actually stepwise addition of two single electrons is the preferred process. The SO_2—molybdic acid reaction has been described by Millard (cited in Mellor's Treatise) as a sensitive test for hypophosphites. Since the product is chiefly H_3PO_3 we suppose that the reduction of H_2SO_3 consists of two consecutive 2 valence changes, the first of which would reduce SO_2 to the 2^+ state (corresponding to SCl_2). In support of this is the fact that $H_2S_2O_4$ and H_2SO_4 as well as S are formed from SO_2 by reduction by H_3PO_2 (cited by Mellor).

With $HMnO_4$, Kolthoff states that H_3PO_2 reacts slowly and incompletely ("Volumetric Analysis", p. 303). This is erroneous. Reaction is virtually instantaneous at room temperature in H_2SO_4 solution, though the solution retains a color slightly resembling permanganate. The spectroscope shows absence of MnO_4^-, the color being due to Mn^{3+}, which liberates much I_2 on adding KI; a precipitate of MnO_2 or Mn_2O_3 appears after about half a minute in N H_2SO_4.

Our study of this reaction shows that when the amount of $KMnO_4$ added exceeds 1 mol per mol of H_3PO_2, 4 equivalents of $KMnO_4$ per mol of H_3PO_2 are consumed (the product being H_3PO_4). When H_3PO_2 is in excess, 4 of the 5 oxidation equivalents of each mol of $KMnO_4$ are reduced, leaving Mn^{3+}; the excess of H_3PO_2 is not rapidly oxidized by Mn^{3+} at room temperature. The rapid process is apparently the transfer of 4 electrons in a single step. If the $KMnO_4$ is first reduced to MnO_2 by adding excess of $MnSO_4$, subsequent addition of H_3PO_2 and heating just to boiling, 2 equivalents per mol of H_3PO_2 are consumed, and H_3PO_3 is formed. Longer heating causes a much slower oxidation of H_3PO_3 to H_3PO_4 by MnO_2. The reactions are in effect the following: $P^{1+} + Mn^{7+} \to P^{5+} + Mn^{3+}$ (rapid); $P^{1+} + Mn^{4+} \to P^{3+} + Mn^{2+}$ (boiling).

There are, of course, many other reactions besides those cited which merit study from the point of view set forth in this paper. We have surveyed a number of others, but only a small fraction of the very large number of possible cases. As

stated in the introductory paragraphs, the author is aware that an adequate test of the principle will require much more extensive and more precise investigation, which it is hoped others besides ourselves will undertake.

If space permitted it would be appropriate to comment here on the applications and implications of the "equi-valence change principle" in biological oxidation, for that is our primary interest. It is believed probable that the principle may be applicable to the behavior of molecular oxygen in auto-oxidation, to induced oxidations, and perhaps to the mechanism of action of biological oxidation catalysts. Brief reference to several of these points has been made in an earlier paper (J. Phys. Chem. *40*, 1021, 1936). Further analysis had best wait until more facts have been accumulated.

POLAROGRAPHIC INVESTIGATIONS OF REVERSIBLE AND IRREVERSIBLE OXIDATIONS AND REDUCTIONS AT THE DROPPING MERCURY ELECTRODE*

Otto H. Müller

For the first twelve years of its existence (1922-34), the polarographic method of Heyrovský was developed empirically. A large number of reducible inorganic and organic substances was found, and convenient though arbitrary methods were developed for their analysis. By 1934, however, some important factors influencing the height of the polarographic wave were recognized by Heyrovský (1), and Ilkovič (2) was able to give a formula for the diffusion current. The reduction and deposition potentials used then showed only a vague relation to the potentiometrically and thermodynamically established normal electrode potentials of the reduced substances. This did not seem unreasonable, because in the usual potentiometric studies one deals with systems at equilibrium, while in polarographic work equilibrium would hardly be expected, since conditions at the electrode are constantly altered not only by the moving surface of the growing mercury drop but also by the current which flows. In addition, the very high overvoltage in the deposition of hydrogen suggested that other reductions at the dropping mercury electrode might also show an overvoltage. However, in 1935, when Heyrovský and Ilkovič (3) recognized the theoretical significance of the half-wave potential and substituted it for the arbitrary reduction and deposition potentials, the close relation of polarographic potentials to the normal electrode potentials became apparent. The half-wave potential, measured at the point of inflection of the polarographic wave, was found by these authors to be a constant for most reactions at the dropping mercury electrode even though the drop-time of the electrode and the concentration of the reducible material was varied. Subsequently, Heyrovský and Ilkovič developed equations covering many types of polarographic curves (3) and anticipated that this half-wave potential should be equal to the normal oxidation-reduction potential of the systems. The experimental proof of this hypothesis was furnished in 1937 by Müller and Baumberger (4) who demonstrated that *in well buffered solutions the polarographic half-wave potential is equal to the potentiometrically established E'₀ of simple reversible organic oxidation-reduction systems.* In more complicated systems, for instance systems which do not come to equilibrium in the absence of a catalyst, or in coupled oxidation-reduction systems, this relation has not been demonstrated; neither have attempts to show this relation for in-

organic systems been entirely successful (5). Nevertheless, there are sufficient simple reversible organic oxidation-reduction systems which obey the above rule that the relation between potentiometric and polarographic potentials has become quite clear. This paper correlates theories and equations developed by the potentiometric method with polarographic studies of reversible and irreversible reductions and oxidations. In addition, a few applications of the polarographic method to the study of biological oxidations will be presented.

The Polarographic Method

Only the essentials of this relatively new method can be described here. Detailed descriptions may be found in the monographs and reviews of Heyrovský (6, 7), Semerano (8), Hohn (9), Kolthoff and Lingane (10), and Müller (11).

Heyrovský was the first to electrolyze solutions between a slowly dropping mercury electrode and a non-polarizable half-cell. Applying different voltages to such cells from a potentiometer and observing the resulting currents on a sensitive galvanometer, he found that by plotting the current against the applied voltage, curves are obtained which, under optimum conditions, indicate the nature and the quantity of many electroreducible substances present in the solutions. Much time can be saved and a continuous current-voltage curve may be obtained with an automatic recording device which synchronizes the change in applied voltage with the movement of a photosensitive paper. Heyrovský and Shikata (12) designed such a device in 1925 and called it a "polarograph", and the current-voltage curves obtained with it "polarograms". While the development of the polarographic method has been closely associated with this instrument, it is obviously not essential, and these studies are possible, though more laborious, with a potentiometer, galvanometer, dropping mercury electrode, and calomel half-cell.

A simple arrangement suitable for such studies is shown schematically in Fig. 1. A student type potentiometer (P) is satisfactory, the galvanometer (G) should have a fairly slow period and a sensitivity of 10⁻⁸ amp. or better. An Ayrton shunt (S) with a total resistance equal to the critical damping resistance of the galvanometer is desirable for modifying the galvanometer sensitivity. The reference cell (R.E.) drawn is a calomel half-cell, but can be any other non-polarizable half-cell of known potential. Connection between the reference cell and the electrolytic vessel is made
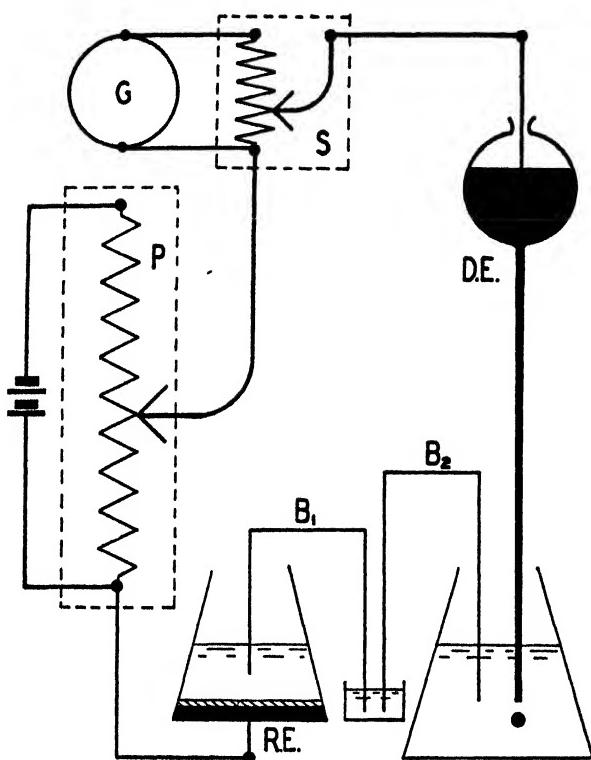
Fig. 1. Simple arrangement for obtaining current-voltage curves with the dropping mercury electrode.

by suitable agar bridges B_1 and B_2 (13). Instead of the separate reference cell, a layer of mercury on the bottom of the electrolytic vessel has been used extensively; the bridges are thus avoided, but the potential of such an electrode differs from case to case and must be measured in each analysis (11).

The dropping mercury electrode (D.E.) makes the method unique, because, unlike all other electrodes used, it has a surface which, at least in part, is fresh at any time during its growth. It furthermore has the important distinction of having a short life-time. This naturally eliminates troublesome aging effects so that the results obtained are perfectly reproducible. All polarographic curves represent results obtained with hundreds of different individual mercury electrodes, and the current corresponding to each applied voltage is well defined and independent of the previous course of the electrolysis and of the length of time that the current has passed. Platinum or gold electrodes often establish a stable potential after several hours in sluggish systems due to the accumulation of electroactive material. This accumulation is impossible on the dropping mercury electrode surface. Only those phenomena which occur during the life-time of a single mercury drop (6-8 seconds in water, 3-4 seconds in

an electrolyte) have an influence on the potential.

Since the life-time of the electrode is so short, only a minute fraction of the electroactive material in solution can be reduced or oxidized at its surface. If 5 cc. of solution is electrolyzed, superimposable polarograms can be repeated many times, demonstrating that the solution as a whole remains unchanged. The current is limited by the quantity of material which can reach the surface of the mercury drop during its existence. This is the basis for quantitative polarography. If surface-active forces and the effect of the electrostatic field on the electroactive material are eliminated by the addition of some indifferent surface-active material (maxima suppressors) and of indifferent or supporting electrolytes, the transport of electroactive material to the electrode depends upon simple diffusion. This diffusion will increase as the gradient from solution to electrode surface is increased by reduction or oxidation of the material. The current obtained when this gradient is maximal has been called the diffusion current.

Ilkovič (2) has derived an equation for this diffusion current which has been verified experimentally:

$$I_d = 0.627 \cdot n \cdot F \cdot D^{1/2} \cdot C \cdot m^{2/3} \cdot t^{1/6} \quad (1)$$

Here n is the number of electrons involved in the reduction or oxidation of one molecule of the reducible or oxidizable substance, F is the Faraday, D is the diffusion constant of the reacting substance, C is the concentration of the reacting substance in the body of the solution, m is the weight of mercury flowing from the capillary per second, and t is the drop-time of the electrode (the latter two quantities have to be determined at the potential at which the diffusion current is measured). In most work calibration curves are obtained with the same electrode which is used for the determinations of the unknowns; the factors m and t will then be constant.

Of course, the diffusion current can not be obtained until the applied voltage is sufficiently negative in the case of an electro-reduction and sufficiently positive in the case of an electro-oxidation. If inadequate voltage is applied, either no current or only a fraction of the diffusion current will flow. If the applied potential is altered until a flow of current becomes just perceptible, the so-called deposition potential of the substance has been attained. However, such deposition potentials are not well defined because they obviously depend on the sensitivity of the apparatus. In the early polarographic work, electro-reducible substances were characterized by potentials which were variously defined (11). The one most used was obtained by the so-called 45° tangent method

(an example is shown in Fig. 2B; an arrow indicates the potential). None of these definitions was entirely satisfactory, however, because the values changed with concentration of the reduced substance and with the drop-time of the electrode. In 1935, this difficulty was finally overcome by Heyrovský and Ilkovič (3) who introduced the *half-wave potential*. This half-wave potential is the point of inflection of the polarographic wave and is the most easily determined point on the curve. It has great theoretical significance which will be discussed later.

Before proceeding further, it is necessary to emphasize that the polarographic curves are not current-*potential* curves, but that they are current-*voltage* curves. It is the applied voltage which is plotted and the applied potential can only be calculated, if the resistance of the system is known, by the simple formula

$$E = V - IR \qquad (2)$$

in which E is the potential, V the applied voltage, I the current, and R the resistance. The factor IR can be neglected *only* when it approaches the limit of error of polarographic measurements, which is 3-10 mv. For instance if R is 1000 ohms (a common value in the absence of agar bridges from the circuit), the polarographic curve may be considered a current-*potential* curve only up to currents of 10^{-6} amp.; at greater currents, IR corrections for potential become necessary.

In order to be sure that the current-voltage curves represent only processes at the dropping mercury electrode, it is essential to have a stable electrode to which all these potentials are referred. A calomel half-cell in an arrangement as shown in Fig. 1 has been found very convenient. All polarograms are then referred to the same electrode and the applied potential values can easily be converted to potentials on the E_h scale by adding the potential of the calomel half-cell. Under suitable conditions, the range of potentials which can be studied polarographically is from $E_h = +0.65$ to -1.60 volts (11, 13). In some special cases -2.3 volts can be reached.

As a rule, solutions are freed from atmospheric oxygen before each experiment by a stream of hydrogen or nitrogen gas, because oxygen is reducible at the dropping mercury electrode (14) and would interfere with most determinations.

Reversible Oxidation-Reduction Systems

The systems considered in this section are reversible in a thermodynamic sense. They can be easily characterized polarographically as will be shown later.

Simple inorganic reductions. Of all the reactions at the dropping mercury electrode, the re-

duction of free metallic ions is the simplest and therefore the most suitable for a demonstration of the fundamental processes at the electrode. Fig. 2 may serve as a typical example.
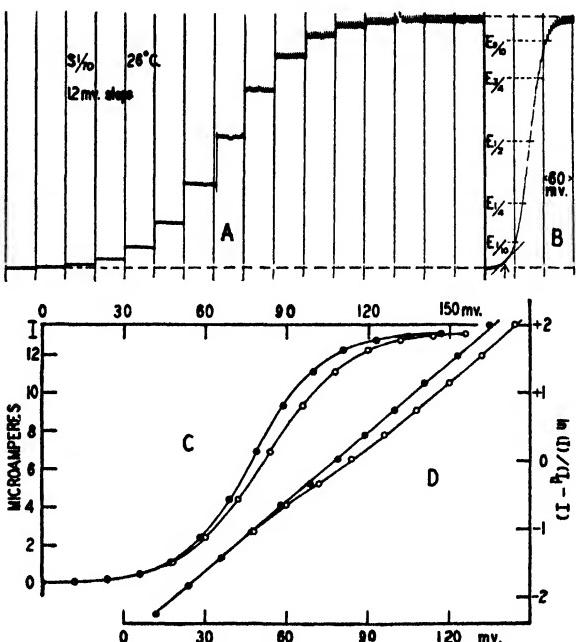
Fig. 2. Curves due to the reduction of cadmium ions.
A. Applied voltage increased in steps.
B. Applied voltage increased continuously (the fractional wave potentials are not yet corrected for IR).
C. ○ Current observed in A plotted against applied voltage.
  ● Current observed in A plotted against applied potential.
D. ○ Graps of ln $(I/I_d - I)$ against applied voltage.
  ● Graph of ln $(I/I_d - I)$ against applied potential.

The curves shown in Fig. 2 are due to the reduction of cadmium ions from an air-free, slightly acid solution of approximately 0.001 N cadmium sulfate in 0.1 N sodium chloride. Curve A was prepared to show what would be observed if the simplified circuit of Fig. 1 were used. Each step represents the current at a constant applied voltage. The increment in applied voltage is 12 mv. between successive steps. The oscillations are due to the increase in current with an increase in drop surface; for convenience the current at maximum drop size is read. The size of these oscillations depends to a large extent on the speed of the galvanometer, since the current starts essentially from zero with the formation of each new drop. The photograph furthermore shows slight irregularities which are due to vibrations of the building causing irregular drop formation. Normally proper cushioning of the electrode and vessels will avoid such complicating factors. In order to make the curve as large as possible on the photographic record, 1/70 of the full galvanometer sensitivity was used, so

that 1 mm. on the polarogram represents a current of 1.4×10^{-7} amp.

The current flow at different applied voltages can be plotted against the applied voltages to produce a "polarogram", Fig. 2C. A photographically recorded polarogram of the same solution is shown in Fig. 2B. The difference between the observed current-voltage and the calculated current-potential curves is demonstrated in Fig. 2C.

The height of the polarographic wave is measured by drawing two parallel lines tangent to the beginning and the end of the wave. This distance can then be subdivided into suitable fractions; lines through these points, parallel to the tangents, will give us the (uncorrected) fractional wave potentials as indicated on the polarogram, Fig. 2B.

Studying the polarograms, Fig. 2B and 2C, we see that at the beginning of the curve the cadmium ions must be present at the electrode interface in the same concentration as in the rest of the solution because the potential is not negative enough to cause any reduction. At the end of the wave, practically all of the cadmium ions which can diffuse to the electrode during its life-time are reduced to cadmium metal which amalgamates with the mercury. During the middle portion of the wave, the condition at the interface can be calculated from the corresponding height of the wave at any point. For instance, at the midpoint, or point of inflection, of the wave ($E_{1/2}$), one half of all the diffusing cadmium ions are reduced at any instant, and the amalgam formed contains half as much cadmium as when all the ions are reduced.

When the current is governed only by the rate of diffusion, which in turn is a function of the difference in concentration between the depleted electrode/solution interface and the body of the solution, it can be expressed as follows (3):

$$I = K \ (C - C_o) \qquad (3)$$

where I is the current and C the concentration in the body of the solution, while C_o is the concentration at the interface. K is the diffusion current constant. When the diffusion current is maximal, C_o, the concentration at the interface, becomes negligibly small compared to C and

$$I_d = K \, C. \qquad (3a)$$

The concentration of the reaction product C_a (in the case of cadmium ions, the amalgam) is also proportional to the current I, and Heyrovský and Ilkovič write

$$C_a = k \, I \qquad (4)$$

where k is again a diffusion constant.

Now if the potential of the dropping mercury electrode during reversible reactions is governed by the same laws as that of other electrodes, the

following formula must hold for all parts of the curve:

$$E = E_o - (RT/nF) \ \ln(C_a/C_o) \qquad (5)$$

if, for simplicity, concentrations may be used instead of activities.

Substituting in this equation from above, we find:

$$E = E_o - (RT/nF) \ \ln(kIK/KC-I) \qquad (6)$$

or

$$E = E_o - (RT/nF) \ \ln(I/I_d-I) + K' \qquad (6a)$$

In the case of the half-wave potential, $I = I_d - I$ so that the logarithmic term drops out:

$$E_{1/2} = E_o + K'. \qquad (7)$$

This means that the half-wave potential of the dropping mercury electrode is equal to the normal electrode potential of the reacting system plus some constant. This constant has been found positive, negative, or zero in metallic reductions, no doubt depending on the nature of the amalgam. However, in all the reversible organic oxidation-reduction systems which have been studied so far, the value of the constant has been zero.

If equation (6a) holds, it is obvious that a graph of E against $\ln(I/I_d - I)$ should give a straight line with a slope equal to RT/nF. This was first tested by Tomeš (15) who found slopes of 0.056, 0.029, and 0.020 v. for mono-, di-, and tri-valent reductions at 20° C. in fair agreement with the theoretically expected values. The polarogram (Fig. 2A and 2C) has been similarly plotted in Fig. 2D and · shows good agreement with the theory. Again the graphs are drawn to illustrate the difference between applied *voltage* and applied *potential*. It may be seen that a straight line is obtained when *potentials* are plotted. Its slope is 0.0295 v. which is the theoretically expected value since the experiment was carried out at 26° C.

Reversible organic oxidations and reductions in well buffered solutions. Organic reactions at the dropping mercury electrode are on the whole much more complicated than inorganic reactions, because the reactants are usually weak acids or bases and because the end product of the electrode-reaction is known with certainty only in reversible systems. However, a reversible organic system in a well buffered solution can be more completely analyzed than an inorganic system, because the end product of the reaction can be added to the solution and its effect studied. The few polarograms of dilute amalgams which have been made (16, 7) show anodic waves of current

with the expected half-wave potentials, but the measurements have not yet been quantitative.

No reversible organic system had been studied until 1937 (4), although much polarographic work had been done on the reduction of organic compounds, *e.g.* ketones, aldehydes, unsaturated acids, and nitro-compounds, the reduction of which is quite irreversible.

The electrode-potential of the reversible organic reaction:

$$Ox + 2\,H^+ + 2\,\ominus \rightleftharpoons Red \qquad (8)$$
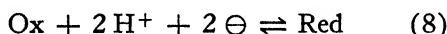
is given by the well-known equation:

$$E_h = E_o - \frac{RT}{2F} \ln \frac{[Red]}{[Ox]} + \qquad (9)$$

$$\frac{RT}{2F} \ln [Ka_1 Ka_2 + Ka_1[H^+] + [H^+]^2]$$

which is written for a two-electron change and in which Ka_1 and Ka_2 are the two dissociation constants of the reductant. Clark and Cohen (17) give a detailed discussion of this and similar equations and their implications. The following are of particular interest:

(a) If the pH is constant:

$$E_h = E'_o - (RT/2F) \ln ([Red]/[Ox]) \qquad (10)$$

(b) If [Red] = [Ox]:

$$E_h = E_o + (RT/2F) \ln [Ka_1 Ka_2 + Ka_1[H^+] + [H^+]^2] \qquad (11)$$

(c) If either Ox or Red is absent, the potential is meaningless because the middle term in equation (9) becomes infinity.

Usually oxidation-reduction potentials are determined by inserting an indicator (so-called indifferent) electrode into the solution and measuring its potential against the known potential of a half-cell which is connected to the unknown by some liquid junction. Any difference in potential between the two half-cells is balanced out by an equal and known counter E.M.F. from a potentiometer, using a galvanometer as null point instrument.[1] Müller and Baumberger (13) have demonstrated that such potential measurements are possible when the dropping mercury electrode is used as indicator electrode. Potentials measured in this way shall be designated as *potentiometric potentials*, to distinguish them from *polarographic potentials* which are calculated from current-voltage curves using equation (2). The fundamental difference between these two terms is that a *"potentiometric potential" represents the electrode potential with respect to the body of the solution, while the "polarographic potential" represents the electrode potential with respect to the interface.* Both will be equal when no current flows, that is, when no reaction takes place at the interface which would make its composition different from that of the body of the solution.

This was demonstrated by Müller and Baumberger (4) in a polarographic investigation of quinhydrone. This compound is easily obtained in a pure form and represents perhaps the best known and most typical example of reversible organic oxidation-reduction systems. A polarogram of quinhydrone in a well-buffered solution is shown in Figs. 3b and 4A. The horizontal, "galvanometer zero" line represents the position of the galvanometer when no current flows. If a substance is reduced and the galvanometer deflection is above this line, the dropping mercury electrode is the cathode; conversely, oxidation of a substance at the dropping mercury electrode, now anode, results in deflection of the galvanometer below this line (4). The currents are cathodic and anodic, respectively. It may be noticed that one-half of the quinhydrone curve is cathodic and one-half is anodic, which would be expected, because quinhydrone in solution dissociates into equivalent amounts of quinone and hydroquinone. At the midpoint of the quinhydrone curve no current flows. This point is the half-wave potential and corresponds to the well-known

---

[1] Using the dropping mercury electrode as indicator electrode and a galvanometer with a sensitivity of 10^{-9} amp./mm./m., it can be shown that the electroactive substances must be present in a concentration of at least 10^{-6} M to poise the electrode; a concentration of similar order of magnitude seems necessary also when platinum or gold electrodes are used. Because only a minute fraction of the solution is electrolyzed in the polarographic method, this poising action does not diminish as rapidly with time as in other electrolyses and can be measured quite accurately.

There has been some discussion in the literature on the advisability of electron-tube voltmeters which will not draw more than 10^{-13} amp. for their operation. While this method no doubt has the advantage that any existing system is much less altered by the flow of current, it has the disadvantage that potentials could be obtained of interfering systems which would have to be present in concentration of only 10^{-10} M. This makes the interpretation of the obtained potentials often doubtful and speculative.
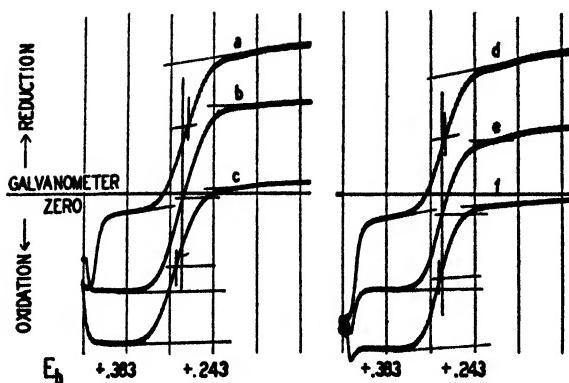
Fig. 3. Polarographic curves of solutions buffered at pH 6.67.

 a. Quinone, Sens. 1/70;
 b. Quinhydrone, Sens. 1/70;
 c. Hydroquinone, Sens. 1/40;
 d. Quinone, Sens. 1/30;
 e. Quinone, partly reduced by platinized asbestos and hydrogen gas, Sens. 1/30;
 f. Quinone, fully reduced by platinized asbestos and hydrogen gas, Sens. 1/30.

E'_0 value that has been obtained with indicator electrodes when the galvanometer serves only as a null-point instrument. This is a case in which the potentiometric potential and the polarographic potential at the half-wave are identical. The *potentiometric* potential can always be read from the polarograms as that point at which the polarographic curve crosses the "galvanometer zero" line.

When only quinone is present in the solution, a *cathodic* wave is obtained (Fig. 3a) which is identical in appearance with a wave due to *anodic* current when only hydroquinone is present (Fig. 3c). The half-wave potentials obtained in

these two curves (corrected for IR) are identical and have the same value as the half-wave potential of the quinhydrone curve which has been shown to be equal to the E'_0 of the system. If solutions in which the composition of the oxidation-reduction system is altered by chemical means (Fig. 3d-f) are studied polarographically, the reversibility of the system can be determined directly: *whenever the half-wave potential is a constant, whether the curve is anodic or cathodic, the system is perfectly reversible in a thermodynamic sense.*

In Figs. 3a and 3c the polarographic potential at the half-wave is different from the potentiometric potential, which means that the interface has a different composition from the body of the solution. The fact that the half-wave potential is equal to the E'_0 indicates further that oxidant and reductant at this point must be in equal concentration in the interface; this is the experimental proof of the conclusion reached by Heyrovský and Ilkovič (3) from a consideration of the geometry of the curve. A further conclusion to be drawn from this experiment is that at the half-wave potential one-half of all the hydroquinone molecules diffusing to the dropping mercury anode during its life-time are oxidized to quinone which must remain long enough at the electrode surface to establish the potential. Similarly at the half-wave potential one-half of all the quinone molecules diffusing to the dropping mercury cathode in unit time are reduced instantly to hydroquinone which remains in the interface long enough to create the condition for the E'_0 (11). After this was established in a number of experiments with other reversible systems (4, 30), it was possible to analyze the composition of the interface at other points along the polarographic curve. It became apparent that the polarographic curve resembled closely a potentiometric oxidative or reductive titration curve (see Fig. 4A and 4C). As may be seen in the case of quinhydrone, the graph of E against $\ln (I/I_d-I)$ gives a straight line with a slope equal to $RT/2F$ (Fig. 4B). This indicates that two electrons are removed or added to the compound in a single step. Instead of $\ln (I/I_d-I)$ one could also write $\ln (Ox_i/Red_i)$ where the subscript i stands for concentrations in the interface. While the two curves (Fig. 4A and 4C) are identical in appearance, they represent two sets of conditions which are considerably different. *In the polarographic curve, only the interface is titrated, and the oxidizing or reducing agent is the electrode itself which removes electrons from or adds them to the electroactive molecules in the interface. In the potentiometric titration the whole solution is changed by some added oxidant or reductant.*

Many other theories and formulas developed in
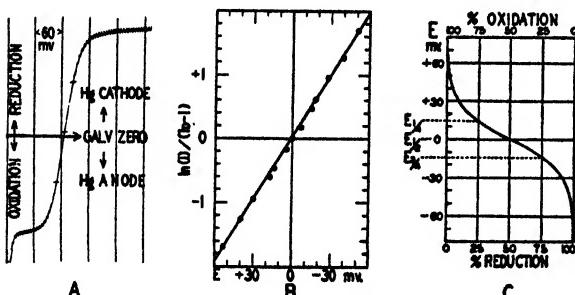
Fig. 4. A. Polarogram of quinhydrone in a buffered solution, representing a direct titration of the electrode-solution interface, while the body of the solution remains practically unchanged.

 B. Graph of $\ln (I/I_d - I)$ obtained from A, against applied potential.

 C. Typical potentiometric oxidative or reductive titration curve representing changes throughout the body of the solution.

the study of electrometric titrations can be applied likewise to polarographic investigations. Typical examples are the following.

As in electrometric titrations (18), it is possible to calculate the theoretical slope of the midportion of polarographic curves. Assuming a constant pH, the equation for the potential is

$$E_h = E'_o - (RT/nF) \ln ([Red]/[Ox]) \tag{10a}$$

or if the degree of reduction in the interface is given by the current I

$$E_h = E'_o - (RT/nF) \ln (I/I_d - I) + K \tag{6a}$$

then

$$dE_h/dI = - (RT/nF) (I_d/I(I_d - I)). \tag{12}$$

When $I = 0.5I_d$ (that is, at the half-wave potential)

$$dE/dI = - 4RT/nF.$$

When $n = 2$ and the temperature $= 30°$ C.

$$dE/dI = - 0.05222.$$

In order to use this method for determining n, sufficient points in the middle of the curve must lie on a straight line so that the tangent can be accurately placed. Secondly, there must not be superimposed upon the conditions of the above equations a change in potential of different source, such as a change of pH of the interface, or a possible semiquinone or meriquinone formation. The pH at the interface could change either by the production of an acid or a base at the electrode in an unbuffered solution or by dilution of the buffer present.

Equation (12) is the reciprocal of the poising effect, i.e. the change in oxidant or reductant with change in the applied E.M.F.:

$$dI/dE = - (RT/nF) (I(I_d - I)/I_d). \tag{13}$$

Differentiating again we obtain

$$d^2I/dE^2 = - (RT/nF) (1 - (2I/I_d)). \tag{14}$$

If we set this second differential equal to zero, we find that the poising effect is maximal when I is equal to $I_d/2$. This poising effect is analogous to Van Slyke's buffer index in acid-base systems (19).

Another way of analyzing for n as well as for possible semiquinones is by means of the "index potential" of Michaelis (20). This is defined as the difference in potential between a system in

equilibrium containing 50 p.c. oxidant and 50 p.c. reductant, and another system containing 75 p.c. oxidant and 25 p.c. reductant or 75 p.c. reductant and 25 p.c. oxidant. At 30° C. in a two-electron change without semiquinone formation, this index potential is 14 mv.

In polarographic work a very similar method has actually been used in determining n for inorganic reductions. Either $E_{1/2} - E_{1/4}$ or $E_{3/4} - E_{1/2}$ has been used to determine n. Thus Tomeš (15) found polarographic index potentials of 27.5 mv. for Tl^+, of 14.4 and 14.3 mv. respectively for Pb^{++} and Cd^{++}, and 9.8 mv. for In^{+++}, which are in good agreement with theory. Similarly the quinhydrone curve (Fig. 4A) gives an index potential of 14 mv. for $E_{1/2} - E_{1/4}$ and of 15 mv. for $E_{3/4} - E_{1/2}$. However, a more accurate determination of n is obtained by plotting the curve of $\ln (I/I_d - I)$ against E_h as has been done in Fig. 4B.

The valency of reduction may be further estimated quickly by measuring the difference between the $E_{9/10}$ and $E_{1/10}$ values (see Fig. 2B) which is equal to $1.9RT/nF$. A useful *approximation* can be obtained by applying the formula $2RT/nF$ to uncorrected current-voltage curves.

Intermediate radicals in reversible systems. The necessity for accurate current-*potential* curves in which every point of the curve is significant becomes very obvious from the above. This is especially important if we wish to consider possibilities which may separate a single polarographic wave into several components due to the formation of intermediate radicals such as semiquinones and meriquinones.

To test the applicability of the polarographic method to the study of semiquinones, Müller and Baumberger (4) investigated rosinduline GG. However, the preparation used was not very pure and, although the curves obtained showed two-step reductions at the proper pH, they were complicated by additional waves due to impurities. Fortunately, I have been able to continue these studies with other semiquinone-forming compounds of highest purity which were kindly given to me by Dr. L. Michaelis.

One of the best of these compounds was α-oxyphenazine. Michaelis has shown (21) that the semiquinone of this compound is the more stable the more acid the solution. This is demonstrated polarographically in Fig. 5, where at pH 2.13 two waves of equal height are seen. In the more alkaline solutions these two waves overlap, and the existence of a semiquinone can only be revealed by an analysis of the index potential as described by Michaelis (20).

The following equation of Michaelis and Schubert (22) describes the potentiometric titration curves during semiquinone formation:

$$E = E_m - \frac{RT}{2F} \ln \frac{1+\mu}{1-\mu} - \frac{RT}{2F} \ln \frac{\sqrt{1 + (4\kappa - 1)\ (1 - \mu^2)} + \mu}{\sqrt{1 + (4\kappa - 1)\ (1 - \mu^2)} - \mu} \qquad (15)$$
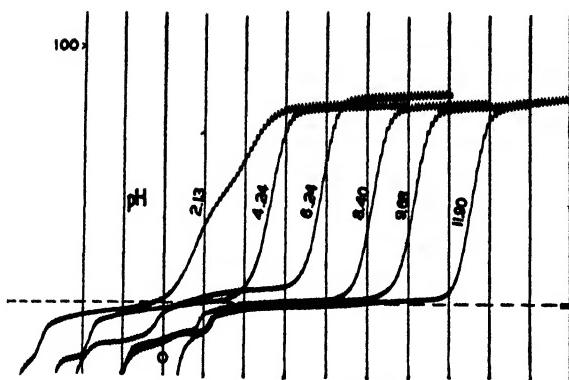
Fig. 5. Polarographic curves of α-oxyphenazine in solutions buffered at different pH. ○ indicates zero applied voltage, referred to the calomel half-cell, i.e. $E_h = 0.242$ v.

This equation has been reinterpreted in terms of polarographic current-potential curves as follows (23):

oxidation or reduction is observed by potentiometric means (22). This may be distinguished from that due to a simple semiquinone by changing the concentrations of the reactants. In reactions at the dropping mercury electrode, in which the semiquinone radical is produced at the electrode interface only during the life-time of a drop, one should expect no difference unless the process of dimerization is as fast as the electrode reaction. Also, the solution would have to be more concentrated than in customary analyses. However, the possibility exists of studying solutions in which, by the addition of a reducing agent, considerable quantities of the meriquinone have been produced.

Reversible organic oxidations and reductions in unbuffered solutions. As is well known, the quinhydrone electrode is used for the determination of pH because its potential is a linear function of pH at constant temperature in the range of pH 1 to pH 8. This dependence is due to the weak acidity of hydroquinone and to the fact that

$$E = E_m - \frac{RT}{2F} \ln \frac{I}{I_d - I} - \frac{RT}{2F} \ln \frac{\sqrt{4\kappa I_d^2 - (4\kappa - 1)\ (I_d - 2I)^2} - (I_d - 2I)}{\sqrt{4\kappa I_d^2 - (4\kappa - 1)\ (I_d - 2I)^2} + (I_d - 2I)} \qquad (16)$$

In this equation E is the observed potential, E_m is the potential when [Ox] = [Red], and κ is the dismutation constant (22) expressing the equilibrium [Ox] [Red]/[S]^2 of the reaction

$$2S \rightleftharpoons Ox + Red \qquad (17)$$

where S represents the semiquinone. This equation (16) has been found to fit polarographic curves (23), again demonstrating the fact that thermodynamic conditions must exist at the electrode interface. While the polarographic method cannot compare in accuracy with the potentiometric method for the determination of some semiquinones, it is useful for orientative studies. However, the dropping mercury electrode offers a definite advantage because it permits the study of potentials and semiquinones in the overvoltage range (23).

Because of the speed of electrode reactions, the polarographic method is of value in the study of intermediate radicals of extreme lability. This will be pointed out in the discussion of irreversible reactions at the dropping mercury electrode.

If the semiquinone radicals combine to form a quinhydrone or meriquinone, again a two-step

in the electrode reaction only the ionic form of hydroquinone can be considered:

$$\text{(18)}$$
$$Q + 2\ominus + 2H^+ \rightleftharpoons Q^{--} + 2H^+ \rightleftharpoons H_2Q$$
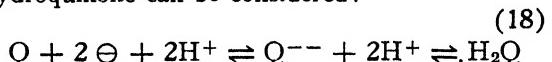
The identity of the half-wave potentials of quinone reduction and hydroquinone oxidation demonstrates that the equilibria shown in this formula must be established with extreme rapidity. The constancy of the pH at the electrode surface implies, furthermore, a similarly rapid dissociation or association of the buffer molecules and ions present.

This last consideration led me to a polarographic investigation of quinhydrone in unbuffered solutions (24). It was found that the smooth single wave obtained with quinhydrone in buffered solutions (Fig. 3b and 4A) broke up into two entirely separate waves when a potassium nitrate solution was used (Fig. 6A). It could be ascertained that these were caused by the reduction of quinone and by the oxidation of hydroquinone, respectively. The half-wave potentials, of course, were no longer equal and did not indicate the pH of the potassium nitrate solution, which was practically neutral. The pH calculated from the
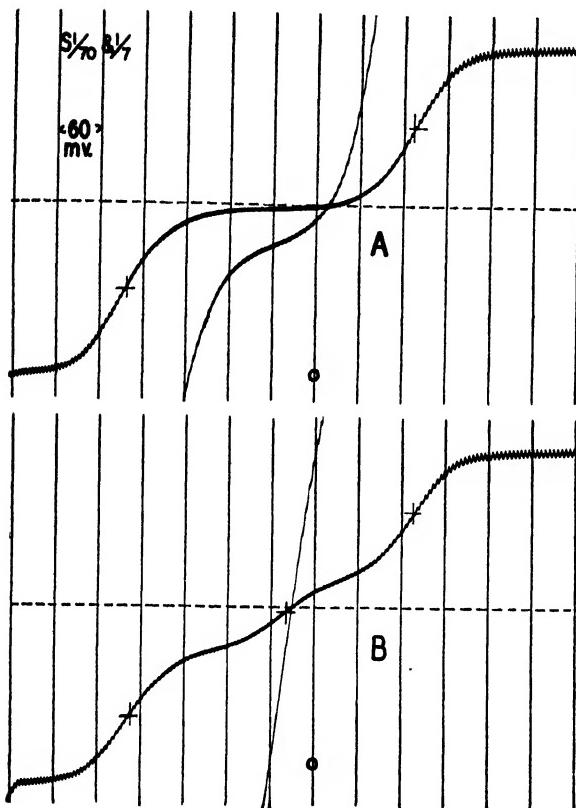
Fig. 6. Polarograms of quinhydrone. ○ indicates zero applied voltage.

A. In unbuffered solution.

B. In slightly buffered solution.

quinone reduction half-wave potential was about 10, and that from the hydroquinone oxidation half-wave potential was about 3. These phenomena could be satisfactorily explained by a consideration of equation (14), where hydrogen ions can come only from water or from hydroquinone.

In the oxidation of hydroquinone to quinone, two hydrogen ions are liberated for each hydroquinone molecule oxidized. In an unbuffered solution those hydrogen ions will remain at the electrode surface, changing the reduction potential by a change of pH. Therefore, when one-half of all the hydroquinone molecules are oxidized, the hydrogen-ion concentration at the electrode surface will be equimolar with the original concentration of hydroquinone at the electrode interface. This has been demonstrated experimentally by Müller (24).

In the reduction of quinone, hydrogen ions are necessary to combine with the hydroquinone ions which are formed. In the absence of a buffer, they must come from water present at the electrode interface, the pH of which is, of course, changed simultaneously. When a pH is reached at which the formed hydroquinone ions are capable

of existing in the ionic state, no more hydrogen ions are needed for the reduction, and the half-wave potential reaches a limit in the neighborhood of the dissociation constant of the reductant in unbuffered solutions (11, 24).

The curve due to quinhydrone in Fig. 6A is not particularly suited for theoretical treatment, because not only is the pH different at the anodic and cathodic half-wave potentials, but also the ratio of oxidant and reductant is not 1:1, but 3:1 for the anodic wave and 1:3 for the cathodic wave. Therefore, another polarogram is presented in Fig. 7a in which only hydroquinone is present in an unbuffered solution. Fig. 7b-g shows the change in this curve when increasing amounts of buffer are added to this solution. In curve 7g, the concentration of buffer is 50 times the concentration of the hydroquinone, so that the solution can be considered well buffered (11). It may be seen by inspection that the curve 7a is not symmetrical with respect to its half-wave potential. Also, plotting $\ln (I/I_d-I)$, a straight line is not obtained. As will be shown, this is caused by the change of pH at the electrode interface due to liberation of hydrogen ions from the hydroquinone which is progressively oxidized to quinone.

We know that the ratio of oxidant and reductant is unity at the half-wave potential, and if it may be assumed that the electrode potential still is defined by the conditions at the interface according to equation (9), we can calculate the pH at that point and use it as reference point for
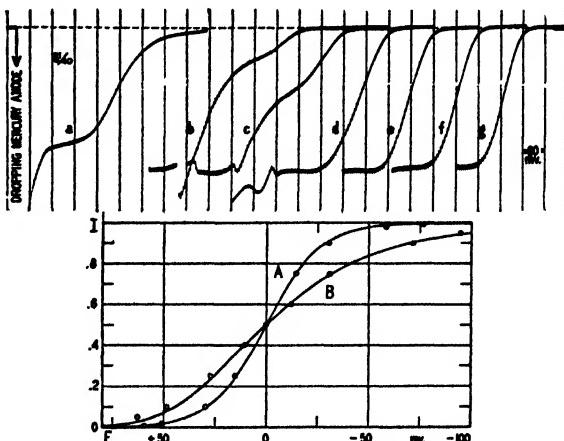
Fig. 7. Polarographic curves of anodic oxidation of hydroquinone.

a. In unbuffered solution.

b-g. In solutions to which increasing amounts of buffer were added.

A. Theoretical curve for well-buffered solution with experimental points from curve g.

B. Theoretical curve for unbuffered solution with experimental points from curve a.

further analysis. Let us call this point E^*_o and define it as the E'_o of a system at a hydrogen ion concentration equal to its own concentration. Using equation (10), we can then construct a theoretical curve for a two-electron reaction at a pH which is constant and which corresponds to this E^*_o. The curve thus obtained is the symmetrical "S"-curve shown in Fig. 7A.

Since the hydroquinone reduction takes place in an unbuffered solution, we know that the pH at the interface cannot be constant and we have to use equation (9). Because Ka_1 and Ka_2 are much smaller than [H^+] in this case, we can neglect these two terms to get

$$E_h = E_o - (RT/2F) \ln ([Red]/[Ox]) + (RT/F) \ln [H^+] \qquad (19)$$

As discussed above, the hydrogen ion concentration is a function of the current, I, so it is possible to rewrite equation (19) to apply to polarographic current-potential curves:

$$E_h = E^*_o - (RT/2F) \ln (I_d - I/I) + (RT/F) \ln (2I/I_d) \qquad (20)$$

This equation is composed of three terms. The first, E^*_o, is dependent on the concentration of the reacting material according to the above definition. The second term describes the normal symmetric "S"-curve in a reversible oxidation at the dropping electrode, while the third term describes the shifts of points of this curve with changing pH. The last two terms are independent of the concentration of the reacting materials and define the shape of the curve in unbuffered solutions. It may be seen that equation (20) reduces to $E_h = E^*_o$ at the half-wave, where $I = 0.5I_d$. A curve calculated from equation (20) has been drawn in Fig. 7B together with the experimental points from Fig. 7a. The good agreement between theoretical and experimental points, plus the fact that the E^*_o varies with concentration (24), may be considered a proof of the validity of equation (20).

Reversing signs before the second and third terms of equation (20) gives an equation which fits the cathodic wave obtained if the reduction of quinone is carried out at such concentrations that the pH created at the interface does not reach the pKa of the hydroquinone. Details of these experiments will be published elsewhere.

When to such unbuffered solutions a suitable buffer is added in a concentration below that of the quinhydrone, a third wave (24) appears between the two waves shown in Fig. 6A, indicating the buffer action at the electrode surface (see Fig. 6B). The sum of these three waves is, of course, the same as the sum of the two waves in Fig. 6A,

since it depends on the concentration of quinhydrone. The trace of buffer added has a certain capacity, limited by its concentration, to keep the pH within narrow limits; when this is exhausted, the pH can change. When the buffer concentration is made greater than that of the quinhydrone, it is able to keep the pH at the electrode interface fairly constant even though all of the hydroquinone be oxidized or all of the quinone be reduced. In this case, the continuous anodic-cathodic curve characteristic of reversible oxidation-reduction systems (Fig. 3b and 4A) is obtained. The buffer action at the dropping mercury electrode provides a new but limited means for the qualitative and quantitative determination of the components of the buffer (24), which otherwise cannot be determined polarographically.

As is well known, buffers have their greatest buffer capacity at a pH equal to their pKa (19). To insure good buffering at the electrode interface, this fact must be kept in mind when choosing the buffer (11, 24). In polarographic studies, it is necessary, furthermore, that the association or dissociation of the buffer molecules and ions be extremely rapid to permit the establishment of the equilibria shown in equation (18) during the reaction at the electrode. Most buffer acids are primary acids in Lewis' sense (25), i.e. acids which dissociate or associate without requiring activation energies. Carbonic acid is the typical example of a secondary acid, since it dissociates very slowly, requiring an energy of activation. In agreement with this property of carbonic acid, it was found polarographically (24) that this acid exerted no buffer action at the dropping mercury electrode for the reactions of quinhydrone. The biological significance of this and its relation to carbonic anhydrase is obvious.

Irreversible Reductions at the Dropping Mercury Electrode.

Most organic reductions which have been investigated polarographically are not of the type mentioned in the preceding section, but are more or less irreversible. Because in these reductions, too, a smooth S-curve is obtained on the polarogram, and a regular shift of the reduction potential with pH is observed, they have often erroneously been called reversible, although the corresponding oxidations of the end-products at the dropping mercury electrode have not been demonstrated. The end-products of such reactions are never known with certainty because not enough is formed at the mercury drops to permit an analysis; but in some instances a number of end-products have been isolated when the electrolysis was continued for a long time and electrodes with large surfaces were used. However, these reduction products cannot be oxidized at the dropping

mercury electrode at the same potential at which they were formed from the oxidant. It must be concluded, therefore, that these processes on the whole must be irreversible in a thermodynamic sense. This conclusion does not eliminate the possibility of a reversible step somewhere in the complicated reduction process, which would be preferentially picked out and represented by the reaction at the dropping mercury electrode. Müller and Baumberger (26) have discussed this possibility in the light of previous electrochemical studies.

Conant (27) studied a number of irreversible reductions which proceed at different rates depending on the oxidation-reduction potential of the system used for the reduction. He concluded that the first step in the reduction must have been reversible and instantaneous, while the next, irreversible, step was slow enough to permit measurement. The irreversible system could be characterized by a potential, the "apparent reduction potential" (A.R.P.). This was defined by Conant as the potential of a "critical reagent" which would just cause "appreciable reduction" (20 to 30 per cent in 30 minutes).

Only a few of the systems described by Conant (27) have been studied polarographically. However, enough material was available to permit a comparison (11, 26) which showed that Conant's A.R.P. more nearly coincided with the "deposition potential" (see p. 60) of the organic substance than with the polarographic half-wave potential. This could be expected, considering the methods of measurement; while Conant measured the A.R.P. indirectly by observing the slow, irreversible process, the polarograph appears to record selectively the reversible portion of the reaction. Müller (11) proposed to designate those half-wave potentials which are not truly reversible, which show dependence on pH, and which demonstrate partial reversibility by means of smooth S-curves as "polarographic apparent reduction potentials" (P.A.R.P.).

The reversible step in these irreversible reactions may be due to a direct electron addition to the organic substance or due to a primary reduction of hydrogen ions to hydrogen atoms which combine with the organic substance. This may be expressed in the following equations:

$$R + 2 \ominus + 2H^+ \rightleftharpoons R^{--*} + 2H^+ \rightarrow RH_2$$

or $$(21)$$
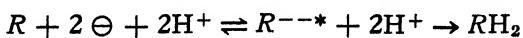
$$R + 2 \ominus \rightleftharpoons R^{--*} \rightarrow R^{--}$$

where R^{--*} is the reduction product in its unstable form which undergoes an irreversible secondary reaction by intermolecular rearrange-
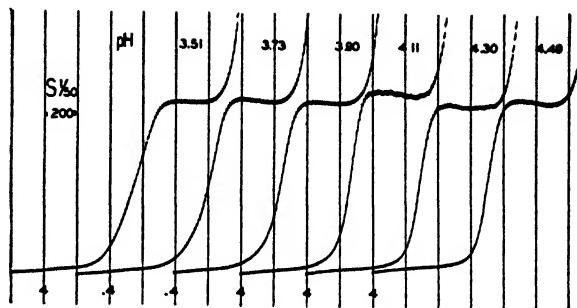
Fig. 8. Irreversible reduction of pyruvic acid at different pH.

ment either before or after the addition of hydrogen ions, and

$$R + 2 \ominus + 2H^+ \rightleftharpoons R + 2H \rightarrow RH_2 \quad (22)$$

No conclusive proof has been advanced that either is the only reaction. Both types of reaction may take place at the dropping mercury electrode, and it may be that pH is the factor governing the predominance of one reaction. Polarogram Fig. 8 demonstrates the reduction of pyruvic acid in well buffered solutions of varying pH. It may be noticed that the degree of symmetry of the S-curves with respect to their half-wave potentials varies with pH. This asymmetry suggested a potential equation similar to equation (20), which fitted the asymmetrical curve obtained in the reduction of a reversible system in an unbuffered solution. However, since the buffering of the pyruvate solutions was quite adequate to prevent changes in pH at the interface during the reaction, some other factor had to be substituted for the pH factor in equation (20) to account for the observed changes in the curves. Additional experiments will be necessary to define this other factor before final interpretations of the reaction mechanism will become possible.

Even in these irreversible reductions it is possible to observe two-step reductions suggesting intermediate radicals which form only at a suitable pH. Tokuoka (28) found that the single benzaldehyde wave broke into two steps of equal height when the pH of the solution was lowered. A similar observation was reported for benzophenone by Schweitzer and Laqueur (29). The simple ratio in these stepwise reductions need not necessarily be unity in all cases, as may be demonstrated by the polarogram in Fig. 9. Picric acid was reduced from well-buffered solutions over the range of pH 2.2 to pH 8. In the more acid solutions two waves are obtained with a relative height of 3:2. The first large wave breaks up into two components of fixed proportions as the solution becomes more alkaline than pH 4. Here

Fig. 9. Step formation in the irreversible reduction of picric acid with change in pH. Each curve starts at zero applied voltage.

the ratio of the two steps is 1:2, so that the reduction at pH 8 goes on in three steps with a ratio of 1:2:2. Similar results have been obtained with other nitrated phenols. The significance of these steps and the number of electrons concerned in each is being investigated.

These reductions in steps of fixed ratio should not be confused with the apparent stepwise reductions which are obtained when, due to pH changes in the solution, a fraction of the reacting substance undergoes a change in structure before it is reduced. For instance, Müller and Baumberger have shown (26) that in the keto-enol tautomerism of pyruvic acid and in the polymerization of this compound, which reactions are governed by the pH of the solution, different waves are obtained for each component of the solution. The relative heights of these waves are not in a fixed ratio, but one wave gradually increases as the other diminishes with changes in pH.

The Polarographic Method in the Study of Biological Oxidations.

The application of the polarographic method to biological studies has so far been very limited. The qualitative and quantitative analyses which were at once suggested when many organic compounds were found reducible in pure solution are not yet possible in unknown solutions, because no detailed studies have been made of the preparation of these solutions for the polarographic analysis. It is, of course, impossible to make a complete analysis of an unknown simply by putting a dropping mercury electrode in the solution and electrolyzing it. There are so many substances with coinciding potentials (11) that with very few exceptions preliminary treatment of the solution is absolutely essential. Metallic ions which may be present in biological fluids are only occasionally liberated from the organic components by acidification of the solution, so ashing of the

substance and subsequent solution in acid becomes necessary before the analysis. Nevertheless, the potentialities of the method cannot be doubted, and it seems like a promising and worthwhile task to develop these biological analyses. Once the necessary conditions for such polarographic analyses are established, it should not be very difficult to find polarometric (31) short-cut methods which could be used for routine work in every laboratory.

In a number of cases the polarograph has already proven of considerable value as a useful analytical tool for the study of biological problems. Two of the most important will be mentioned in the following, together with some speculations which are based on the interpretations of polarographic curves.

Dissolved oxygen can be determined polarographically because oxygen is reduced directly at the dropping mercury electrode and the resulting current is proportional to its concentration. This reduction of oxygen has been known for a long time; in fact, it brought about the development of the polarographic method (6). It has been studied in detail by Vítek (14), and simplified applications have been devised by Müller and Baumberger (32) and by Petering and Daniels (33), permitting the analysis of dissolved oxygen to 0.02 parts per million by weight. Oxygen consumption curves in homogeneous solutions are easily and automatically obtained with the polarograph (see Baumberger, this Symposium).

A reaction entirely different from those considered in this paper has become well known and is being used for determinations in biological fluids. This reaction has been developed by Brdička (34); it is characteristic of sulfhydryl groups and depends on the catalytic deposition of hydrogen from this group when it is present as an organo-metallic complex (usually as the cobalt-complex). It has found a wide application for the determination of cystine, cysteine, and sulfur-containing proteins, and is being considered as a means for the diagnosis of cancer (35).

In discussing the pH of the living cell, Spek (36) has pointed out that different parts of the plasma in the same cell have pH values which may vary from pH 5.0 to pH 7.8, and that two types of differentiation may be determined entirely by the pH of the different cell substances. In one case certain cell substances are accumulated underneath the whole cell membrane, while the other components move away from the surface. In spherical cells, a sort of concentric cell-structure results. In another type, a part of the cell substances moves to one "pole" of the cell and the other to the opposite side. This results in "bipolar differentiation". According to Spek (36), vital staining with indicators has demon-

strated that these intracellular movements show gradations in pH either from the membrane to the center, or from one pole to the other; grada-tions in the oxidation-reduction potential inside the cell have been observed also. As we have seen in this paper, polarographic current-voltage curves demonstrate that pH values at an inter-face can be quite different from those in the body of the solution as the result of a reaction. The effect of buffers on this pH change at the inter-face has also been shown. Using this system as a model, one can visualize cell surfaces at which reactions go on to produce enough acid to exhaust the buffering capacity of the solution and so to cause a marked change in pH. As a consequence the reaction may be decreased in rate, or stopped entirely, or shifted in a new direction.

Outside of the change in activity of enzymes which is brought about by changes in the pH of different portions of the cell, changes may occur in the structure of the reacting substances, such as the keto-enol tautomerism of pyruvic acid (26). This acid holds a key position in practically all schemes of carbohydrate metabolism, in the intermediate steps of which the enol form of pyruvate is often postulated. Müller and Baum-berger (26) have made a polarographic study of the keto-enol tautomerism of pyruvic acid in buffers of different pH. They found that the ratio of keto/enol decreases logarithmically with an increase in pH and that this ratio is unity at pH 5.8. There has been no other satisfactory method for the determination of this enol form, since the usual methods have failed in this case. Nevertheless, the enol form of pyruvic acid has been considered essential in the decarboxylation of pyruvic acid (37) and in the oxidation of pyruvic acid to oxalic acid (38). Furthermore, the phosphopyruvic acid obtained from phospho-glyceric acid is considered to be in the enol form and the enzyme which brings about this rearrange-ment has been called enolase (44). The support-ing evidence usually cited in these cases is the spectroscopic work of Henri and Fromageot (39). This is surprising since Fromageot *et al.* (40) have reinterpreted this paper, proposing a different compound in place of the enol form of pyruvate. According to Müller and Baumberger (26), however, these authors did not go to short enough wavelengths to measure the $C = C$ bond, and their results indicate the degree of poly-merization rather than the degree of enolization.

The marked changes in keto-enol ratio of pyruvic acid observed by Müller and Baumberger fall in a pH range which may be considered physiological if Spek's limits (36) are accepted. This suggested the possibility that these changes may determine the direction of the cell re-actions in which pyruvate ion is involved. If it

be granted that decarboxylase acts only upon the enol form (37) and that lactic acid dehydrogenase acts on both forms of pyruvic acid (41), the pH will play a part in determining the relative pro-portion of carbohydrate metabolism that goes over the two routes (a) and (b):

(a) *Irreversible*:

$$CH_2 = COH - COO^- + H^+$$

<div align="center">decarboxylase</div>

$$\longrightarrow$$

$$CO_2 + CH_3CHO.$$

Reversible:

$$CH_2 = COH - COO^- + 2H^+ + 2\ominus$$

<div align="center">lactic acid
dehydrogenase</div>

$$\rightleftharpoons$$

$$CH_3 - CHOH - COO^-.$$

(b) *Reversible*:

$$CH_3 - CO - COO^- + 2H^+ + 2\ominus$$

<div align="center">lactic acid
dehydrogenase</div>

$$\rightleftharpoons$$

$$CH_3 - CHOH - COO^-.$$
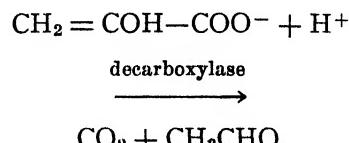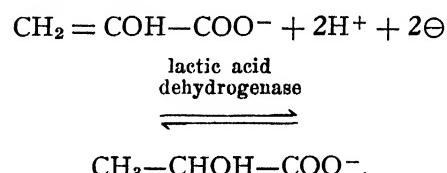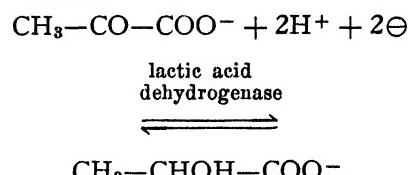
Whenever these reactions occur in the cell, an accumulation of CO_2 with a lowering of pH re-sults unless the buffering and/or the diffusion of the CO_2 into the circulating blood is adequate to prevent this change of pH. The percentage of enol pyruvate in this more acid environment would become smaller, and the carbohydrate breakdown would go largely to lactic acid.

Another point of theoretical interest with regard to coupled oxidation-reduction systems (41) may be mentioned here. The polarographic apparent reduction potential of pyruvate ion is -1.0 volt at pH 7 (26). Barmore (43), using Conant's method (27), found the apparent oxidation po-tential of lactate equal to $+1.0$ volt. These two potentials are a total of two volts apart, and yet by means of suitable enzymes and dyes it is possible to bring them together and to make this system reversible in a thermodynamic sense, so that significant oxidation-reduction potentials ($E_h = -0.186$ v. at pH 7 (42)) can be de-termined. Such potentials are obtained with platinum or gold electrodes, but they can also be obtained with the dropping mercury electrode when it is used as an indicator electrode (13).

However, a polarogram of such solutions shows no smooth S-curves of the type demonstrated for quinhydrone, but only an almost straight line which passes through the galvanometer zero near the expected potential—a fact which may be related to the sluggishness of the systems involved. Furthermore, the wave due to the pyruvate reduction which is obtained in pure solutions disappears in this case. A detailed study of other similar systems in which the enzyme can be prepared in the purest form and in which the relative concentration of the other components can be varied conveniently should be most informative, because it might show the interrelation between enzyme, dye, and oxidizing and reducing components of the systems.

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DISCUSSION

Dr. Barron: Do you think the lactic-pyruvic system is irreversible or reversible?

Dr. Müller: In the absence of enzymes and other catalysts I think it is irreversible in a thermodynamic sense; nevertheless, in this case it is possible to get lactic acid from pyruvic acid, and *vice versa*, if sufficient energy is available to change the compounds into their activated, labile states. Naturally the activated states of lactic acid and of pyruvic acid are not necessarily the same; thus the routes of the forward and backward reactions may be different, as well as the necessary activation energies.

Dr. Barron: The catalyst in the lactic-pyruvic system, as was shown by Borsook and Shott in their determinations of free energies of succinate-fumarate from heat measurements and from potentiometric measurements, does not take up energy. What we were measuring there was the potential energy of the reversible lactic-pyruvic system. I think, therefore, if you do use pyruvic-lactic acid you ought to get a middle potential which ought to coincide with the potential we obtain in the case of an enzyme, because if you are able to determine potentials of even irreversi-

ble systems you ought to be able to determine potentials of reversible systems where the role of the catalyst is to make it measurable in part.

Dr. Müller: Since the polarographic apparent reduction and oxidation potentials do not necessarily represent the same reaction routes which are taken in the presence of a catalyst, there need not be a relation between these potentials and that of the system when it has been made reversible. That the dropping mercury electrode gives the same potentials as a platinum electrode when reversible systems are measured has been shown by Müller and Baumberger.

Dr. Ball: I understood you to say that the polarographic method was useful only in the range of $+0.500$ volt to -2.3 volts, and yet you stated that oxygen was reduced at the dropping mercury electrode. An oxygen electrode has a theoretical value of $+1.23$ volts.

Dr. Müller: We are dealing here with an oxygen overvoltage on the dropping mercury electrode. The reduction of oxygen on our electrode takes place at $E_h +0.200$ v., that is at a potential near that of the calomel electrode, and is but little influenced by pH. This fact is quite well established and should be kept in mind when biological oxidations are discussed. It seems to me just as important as the well known hydrogen overvoltage.

Dr. Ball: Then you are using the European system of nomenclature and not Lewis'?

Dr. Müller: I am using the European system which is almost universally used in American journals where such oxidation-reduction systems are described. Under optimum conditions we can then work in a range of potential from $E_h +0.65$ to -2.3 volts.

Dr. Ball: Is that range independent of pH?

Dr. Müller: No. In buffered solutions especially, the reduction of hydrogen ions to hydrogen gas limits our measurements. At pH 7 our limit is around $E_h -1.4$ volts.

Dr. Shaffer: Is it possible that the upper limit on the positive side of the mercury electrode could be due to its behaving at that point as a mercuric oxide mercury electrode, which changes in a characteristic way with the pH? And would it not be perhaps in that range?

Dr. Müller: Only in alkaline solutions do we have a mercuric oxide electrode as a limit due to the reaction of the mercury ions with the hydroxyl ions; this limiting potential is about -100 mv. from the normal calomel potential. This and other limits can be calculated, as Müller and Baumberger have shown, from the solubility product of the slightly soluble mercury salt.

Dr. Ball: Did I understand you to say that one factor affecting your potentials was an absorption on the surface, and if so will you tell us something more about that?

Dr. Müller: Absorption does not affect the potentials, but it does change the height of the waves. Characteristic maxima appear which indicate that the reducible substance must be present at the interphase in greater concentration than could be expected from simple diffusion. These maxima can be suppressed by the addition of non-reducible, surface-active materials. They can further be correlated with irregularities on the well-known surface tension-potential curves and a definite distinction between positive and negative maxima is possible. This has led Heyrovský to postulate a theory of adsorption, while Antweiler thinks that a stirring effect of the growing mercury droplet is the cause. Perhaps both theories are correct but at present the experimental data are still too sketchy to permit a clear formulation.

Dr. Ball: Would you be able to recognize an effect readily in a complicated system such as, perhaps, the nitrophenols you mentioned?

Dr. Müller: Yes. In that case maxima exist at pH 2 and 3 (see Fig. 8) but not at higher pH. It is best to suppress them if possible.

Dr. Warren: I have been thinking of the capillary electrometer and of change in the surface tension of mercury that occurs when an E.M.F. is applied. In this instrument does that not effect the size of the drop? Does the drop size vary with the E.M.F. and cause some sort of distortion in the curves?

Dr. Müller: Yes, it does. This is a point which must be, and which has been, considered in all good polarographic work. It means a gradual decrease in the height of our waves as the potentials become more negative.

Dr. Barron: Müller has shown us the close correlations between the potentials obtained with the ordinary method in electroactive systems and the potentials obtained in a dropping mercury system. I would like to know what his experience has been in the case of sluggish reversible oxidation-reduction systems, like ascorbic acid, and glutathione, pyridine nucleotides and possibly diphosphothiamine.

Dr. Müller: I have made only a preliminary study of ascorbic acid. It gives good and reproducible curves. When the half-wave potentials are plotted against pH, a curve is obtained with an inflection around pH 4 or 5; but the potentials do not at all agree with the values found by Ball. There is a constant difference of about 200 mv. This discrepancy is outside the experimental error so it is very important to find the reason for it. Perhaps the ascorbic-dehydroascorbic acid system is not quite reversible, but not as irreversible as lactic-pyruvic (in the absence of a catalyst); or perhaps the sluggishness of the system is the cause. I am planning experiments to clear up this question, if possible.

OXIDATION-REDUCTIONS IN HETEROGENEOUS SYSTEMS

Irvin M. Korr

Despite their great biological importance, oxidation-reductions in heterogeneous systems have remained a very neglected field of investigation. There is a conspicuous paucity of papers on the subject; my contribution to the field consists only of a preliminary investigation, carried out nearly four years ago, which I have not had an opportunity to continue. Since the subject is still in a highly speculative stage of development, I feel that I can best serve my function, as a biologist, in this Symposium, by injecting something of the biologist's viewpoint. There is very little more that one can do at this time; one cannot very well present a critical review of data which are not yet available, but one can borrow a few lessons from other fields and outline some of the probable lines of development.

The biologist's interest in heterogeneous systems and in surfaces is certainly well justified. Probably three of the most distinctive characteristics of living matter (that is, of cells) are: First, the colloidal, extremely polyphasic state of protoplasm itself, with its great multiplicity of surfaces. It is undoubtedly the properties of the films, membranes, fibers, capillaries, etc., in which so much of the cell's substance and energy are accumulated, which make possible, within a cell, the maintenance of "sinks and sources of energy", and the tremendous variety of reactions and processes, each apparently independent of all others yet all progressing in coordinated fashion. Together with the second of the three characteristics, namely the enzymes, they make possible the production of substances, chemical changes and other phenomena which the chemist, working with a wide range of temperatures and a great variety of solvents, can only begin to duplicate. Third, is the plasma membrane which determines what shall enter the cell and at what rate, and which is also the seat of so many important electrical phenomena. The essential feature of each is surface.

The past few years have seen great advances in the chemistry of biological oxidation-reductions. The mechanisms by which electrons are transferred from substrate hydrogen to molecular oxygen have been greatly elaborated; a vast variety of cellular components including enzymes, coenzymes, substrates, intermediates, and mediators participating in the oxidation-reduction continuum of the cell have been extracted, purified, crystallized, analyzed and some even synthesized; their potentials have been measured; their reactions under various experimental conditions have been described; they have been fitted into schemes and given their places in the electron path.

It is not always kept in mind, however, that the normal medium for biological oxidation-reductions and for all these participating substances is not pure buffer solution, but protoplasm—with its complexity, heterogeneity and organization. It is true that the coordinative outlook has been developing in biochemistry, together with the strong realization that cellular respiration cannot be understood apart from cellular organization, but unfortunately such awareness is usually accompanied by the pessimistic view that the protoplasmic environment introduces complications hardly possible of formulation.

It is true, of course, that even simple colloidal systems represent a much higher level of integration and complexity than do homogeneous solutions, and that, because of the quantitative and qualitative modifications which interfaces and their molecular groupings impose, there emerge new classes of phenomena for which there are no analogies in homogeneous systems, and which, therefore, require new sets of rules. But it is not premature, with our present knowledge of oxidation-reductions and surface chemistry, meagre as it may be, to begin the formulation of these rules.

I am aware, of course, that considering protoplasm merely as a colloidal solution is not enough; one must also take into consideration cellular architecture and the intracellular organization of surfaces, which may in large part be responsible for the integration of cellular activities and their direction. I believe, however, that the surface approach will be a fruitful one, in that interfaces and the molecular orientations which occur at them represent the first and simplest meeting place between chemistry and gross structure, between the sciences of matter and the sciences of form. In addition, so many of the phenomena appearing at these interfaces, but not in simple solutions, are again found in living systems.

I plan in this paper to consider oxidation-reductions occurring in simple heterogeneous systems, as a start in the formulation of the new rules. I will outline some of the ways in which interfaces may influence oxidative reactions, indicate some of the new phenomena, and finally and briefly, consider the relation between cellular organization and cellular respiration. Exhaustive theoretical treatment and review of the literature have not been attempted; the literature has been drawn upon mostly for illustration. Although a large amount of speculation in this field at present is unavoidable, I have endeavored to restrict the speculative impulse of the biologist as closely as possible to the observed facts.

Possible ways in which interfaces may influence oxidation-reductions. *1.* Adsorption at a phase boundary may completely remove a substance

from reaction by inactivation, *e.g.,* if the bonds of adsorption affect those groups involved in the oxidation-reduction process.

2. Shifts in equilibria may occur in consequence of changes in dissociation constants, differential adsorption of the components of a system, etc.

3. Reaction rates may be modified in consequence of concentration changes, molecular orientation, or real changes in reactivity, etc.

4. The presence of two or more kinds of interfaces may prevent the interaction of systems which ordinarily react in homogeneous solutions, by localizing them on separate phase boundaries or in separate phases.

5. The surfaces may themselves actively participate in the oxidative reactions in many ways. (In this category phenomena of activation and specific catalysis will not be considered.)

The effects produced depend, of course, on the nature of the interfaces and the phases which they separate, the nature of the substances adsorbed, and the nature of the bonds of adsorption.

Although each of the above phenomena, and others, will be considered in the following discussion, they will not, in every case, be taken up separately, since they are all closely interrelated.

Changes in Equilibrium. According to the principles enunciated many years ago by Thompson and by Gibbs, chemical equilibria will be displaced in the direction favoring those substances which lower the surface energy, *i.e.,* those which are positively adsorbed. As stated by Adam:

"If an equilibrium exists in solution between two or more constituent substances, and one of these is adsorbed more strongly than another, that one will be more concentrated in the surface and the equilibrium in the surface layer will be shifted in the direction of that constituent. It often happens, owing to electrolytic dissociation or to hydrolysis, that a single pure substance when dissolved in water consists of such an equilibrium mixture, and if the bulk solution alone were under consideration, an aqueous solution of such a substance would naturally be treated, according to the phase rule, as a two-component system. But when surfaces enter into consideration, unless the ease of adsorption of both the constituents of the equilibrium mixture in solution is identical, the adsorption of each has to be considered separately and consequently the system must be regarded as consisting of three components at least, not two." (2)

One of the most interesting types of equilibrium shifts due to adsorption is that which has come to be known as hydrolytic adsorption. The basis of this phenomenon is that the undissociated form of almost any organic electrolyte dissolved in water is more strongly adsorbed at most interfaces between the aqueous solution and other phases (of lower dielectric constant than water) than is its organic ion. One case is that of soap solutions made by dissolving carefully neutralized soap in water, in which it is found that the undissociated fatty acids are concentrated at the air-liquid surface. That is, the fairly small amounts of hydrolysis in the interior are very much magnified by the great difference in adsorbability between the neutral soap and the free fatty acid.

These shifts in dissociation equilibria are further demonstrated in the experiments of Hartridge and Peters (36, 60) on the influence of pH (of the aqueous phase) on the interfacial tensions between benzene solutions of long-chain acids or amines and aqueous solutions. Close analysis of their tension-pH curves showed the extremely important fact that, at the interface, dissociation of the carboxyl group begins at about 3 pH units to the alkaline side, and of the amine group some 4 units to the acid side of the point at which the corresponding water soluble acid and base would begin to ionize when in solution. That is, it seems that at the interface dissociation constants are shifted in such a direction that diminished dissociation occurs. Since the pK of both acids and amines is shifted towards neutrality, much more dissociation of these compounds must occur within cells than would be expected at the intracellular pH (on the basis of homogeneous solution).

Shifts in dissociation equilibria were strikingly demonstrated in the experiments of Deutsch (24) in which shifts were directly and visibly observable, since the electrolytes in this case were pH indicators. If an indicator which is an acid is dissolved in buffer solution slightly on the alkaline side of its usual color change, and if the solution is then shaken with an immiscible liquid hydrocarbon such as toluene or benzene, the interface of the temporary emulsion becomes strongly colored with the acid form of the indicator. Conversely, a basic indicator dissolved in buffer slightly on the acid side of its turning point, shows the alkaline form of the indicator when a large amount of interface is produced in solution. These color changes correspond to quite substantial shifts in pH, 1 to 1.5 pH units. However, Deutsch, and also Freundlich (31), are convinced that real pH differences between bulk and surface phases are not involved, because the solutions were well buffered and because shifts in either direction could be caused at the "same" interface (benzene-buffer or toluene-buffer), depending upon the acidic or basic nature of the indicator.

On the basis of Peters' (60) experiments, Danielli (23) was led to investigate the possibility that the pH in the surface layer may not be the same as in the bulk aqueous phase, and he adduced considerable evidence that the reaction in the surface phase may be as much as $\pm$ 2 pH units different from that of the aqueous phase. It is shown that dissociation constants in the surface phase are much less than those in the bulk phase owing to the lower dielectric constant in the interface and because of the fact that the ionizable

groups are close enough together to cause a repression of ionization, according to the principle suggested by Bjerrum in 1923. According to Danielli (23), Deutsch and Freundlich were in error in conceiving the difference between the interfacial pH and the bulk reaction as a pre-existing quantity, independent of the adsorption of an indicator. It is his interpretation that, just as in his experiments with simple acids the adsorbed components of the interface are important variables in the determination of this pH difference, so will the adsorption of an acid indicator at an interface tend to produce a displacement of the pH at a surface to a more acid reaction; with a basic dye, the pH change should be in the opposite direction. Thus the results of Deutsch are consistent with the view that the pH of the surface phase is different from that of the bulk, provided due account is taken of the fact that the indicator is concentrated in the interface, and that as a result its influence is not negligible, as in the bulk phase where the indicator will be in very dilute solution.

The corollaries and theoretical implications of these surface phenomena concerned with dissociation and hydrogen ion concentration are, from the point of view of oxidations (as well as from other viewpoints), so vast that we can only begin to appreciate them with our present knowledge of the nature of intracellular interfaces and the changes that take place upon them. However, we may indulge in a certain amount of reasonable speculation.

One important corollary is that intracellular chemical reactions can take place at a variety of hydrogen-ion concentrations at the same time, despite the constancy and uniformity of reaction of the bulk of the fluid contents of the cell. A range of hydrogen-ion concentrations corresponding to at least 4 pH units is thus made possible. Danielli has found that proteins assume strikingly different properties at interfaces as a result of the pH shifts. The importance of these facts to the kinetics of intracellular enzyme reactions is evident.[1]

Of even greater interest in this connection are those oxidative systems in which the energy of dissociation comprises an important fraction of the over-all change in free energy of the oxidation-reduction. Reference is made here particularly to such systems as the reversible dye systems and intracellular hydrogen carriers, and reversible substrate-dehydrogenase systems. One or more dissociation constants are involved in their electrode equations, and their characteristic potentials

are determined over wide ranges by the hydrogen-ion concentration of the medium. With the demonstration that not only pH, but also pK is profoundly modified at interfaces ($K_{surface}/K_{aqueous}$ is 3.1×10^{-2} and 9.3×10^{-2} for palmitic and oleic acids respectively (23)), the range of potentials which a single diffusible carrier can have within a single cell is tremendously extended. It certainly becomes hazardous to estimate the "functional" potential(s) of a given system in the cell on the basis of its behavior in homogeneous buffer solution. The positions of two systems on the potential scale may even be reversed upon adsorption, in a fashion similar to that outlined by Clark (18, p.89) for two systems, the E'_0:pH curves of which cross.

Differential Adsorption. As a result of differences in ionic dissociation, chemical structure, differences in charge, solubility, etc., the oxidized and reduced forms of the same system may show marked differences in adsorbability. One naturally occurring system in which this has been observed is cytochrome-c, the reduced form of which shows only a slight tendency to be adsorbed on kaolin or silicic acid, whereas the oxidized form is strongly adsorbed (73).

As a result of this preferential adsorbability, the concentration ratios of the oxidant to reductant may be very different at the interface from those in the bulk phase. Because of the fact (to be discussed later) that the adsorbed molecules do not so readily exchange electrons with a noble metal electrode immersed in such a system as do dissolved molecules, if at all, the electrode potentials will be much at variance with those calculated from the ratios of total oxidant to total reductant.

Marked shifts in potential can, indeed, readily be demonstrated in simple heterogeneous systems by adding, in nitrogen, the finely divided solid phase suspended in buffer, to a dye system, at a given percentage reduction, dissolved in the same buffer, and by measuring potentials before and after addition. Such a series of exploratory experiments has been reported by Korr (44).

Five substances were investigated: methylene blue, potassium indigo-disulfonate, phenol indophenol, toluylene blue and pyocyanine. The adsorbents included cellulose, kaolin, silica and alumina gels. All preparations were very thoroughly washed before use, both by extraction with water in a Soxhlet extractor and by several days' dialysis against distilled water. All solutions and suspensions were made up, at different pH values, in buffers recommended by Clark (17), and tested, in each experiment, with the quinhydrone and glass electrodes.

For an experiment, each of four electrode vessels of a convenient type previously described (41), and containing 30 ml. of buffer was thoroughly deaerated by a rapid stream of pure nitrogen. When ready, 2 to 4 ml. of the dye (0.0006 M to 0.002 M), reduced to within 25 to 75 p.c. were added from the burette to each of the four electrode vessels. The potential in each was measured over a period of several minutes to make

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[1] As pointed out by Danielli, however, since the spatial range of the pH effect at the surface is small relative to the diameter of a protein enzyme, the latter must be adsorbed with correct orientation in order that the active centers fall within the region of abnormal pH.

certain that it remained at a steady level. Approximately 1, 2 or 3 ml. of the adsorbent suspension (which had been made up in the same buffer solution as the dye, and which had been completely deoxygenated by alternate boiling under reduced pressure and flushing with nitrogen), were transferred, with rigorous exclusion of air, to one of the electrode vessels. To another was transferred a similar quantity of buffer which had been separated from the adsorbent suspension, to control for dilution effects and for any substances which may have been washed off the adsorbent. Potentials were then followed in the experimental and control vessels until steady levels were reached. Several portions of adsorbent and buffer were added, and the potentials followed after each addition. The whole experiment was then repeated on the remaining two electrode vessels. Pure nitrogen streamed through all the electrode vessels, and through the two containers of adsorbent and buffer throughout the experiment.

Since the magnitudes of the effects to be described here are dependent upon the amount of adsorbent added and upon the poising factors (concentration of dye and per cent reduction), the absolute value of a potential shift in a given direction is of little significance. Moreover, due to difficulties in measuring out identical amounts of adsorbent to duplicate vessels, these duplicate experiments rarely agreed in amount of shift, but always agreed with respect to presence or absence of change, and its direction. In Table I, which summarizes the results of these experiments, only the direction of a shift (when it occurs) and the order of magnitude are given for the experimental tube (with adsorbent), as compared with the control tube (without adsorbent). Only in the case of alkaline methylene blue did dilution of the dye with buffer change the potential (20); and this has been allowed for in the tabulation. Small changes of less than 2 mv. are omitted (designated 'no effect'). Amount of adsorption was judged by depth of color on the adsorbent.

TABLE I

Cellulose

Methylene blue	pH 9.0	Negative shift 8-10 mv. Much adsorption.
Toluylene blue	pH 5.9	Negative shift 2 mv. Slight adsorption.
K-indigo-disulfonate Phenol indophenol Pyocyanine	pH 5.9, 9.0	No effect. No appreciable adsorption.

Kaolin

K-indigo-disulfonate	pH 5.7, 9.0	No effect. No adsorption.
Toluylene blue	pH 5.7	Negative shift 10 mv. Much adsorption.
Pyocyanine	pH 9.0	Blue and colorless forms predominate. No effect. No adsorption.
	pH 2.36	Green and red forms predominate. No effect. No adsorption.
	pH 2.36	Green and colorless forms. Positive shift 8-13 mv. Very slight adsorption; deepening of green on exposure to air.
	pH 4.85	Green and colorless forms predominate. Positive shift 6 mv. Very slight adsorption; deepening of green on exposure to air.
	pH 5.0	Blue and colorless forms. Positive shift 10-20 mv. Colorless form adsorbed, passing through green and red forms on exposure to air (see p. 79).

Alumina Gel

Pyocyanine	pH 2.21, 4.70, 5.8 and 9.0, various levels of reduction. No effect. Very slight or no adsorption.
K-indigo-disulfonate	pH 9.1	No effect.
	pH 5.8	Negative shift 6 mv. Slight adsorption.

Silica Gel

Pyocyanine	pH 2.21, 4.70, 5.8, various degrees of reduction. No potential shifts.
	pH 9.1	Positive shift 4-8 mv.
Phenol indophenol	pH 5.8	No effect.
	pH 9.1	Large positive shift, but E.M.F. unsteady.
Toluylene blue	pH 1.36	No effect. Much adsorption.
	pH 5.7	No effect. Much adsorption.
K-indigo-disulfonate	pH 5.8	Positive shift 5 mv.
	pH 9.1	Positive shift 10-20 mv. Much adsorption of leuco form.
Methylene blue	pH 2.21	Negative shift 13 mv. Much adsorption.
	pH 4.70	Negative shift 30 mv. Much adsorption.

It is clear from Table I that a given ratio of oxidant to reductant which gives a certain potential at a platinum electrode may give quite a different one in the presence of adsorbents. The amount of difference is small in most cases, but far in excess of the limit of error, and corresponds to quite significant changes in the Ox/Red ratio. The direction of the difference for a given dye depends on the adsorbent and on the pH, and is always in the direction to be expected from the observed adsorption. For a given combination of dye and adsorbent, the shift may be positive at one pH and negative at another, due to changes in relative adsorption of oxidant and reductant. In the system toluylene blue and silica gel the absence of potential shift, despite marked adsorption, is presumably due to equal adsorption of oxidant and reductant.

It is of interest, also, that the quinhydrone system was affected by interfaces, as indicated by apparent shifts in pH of buffers, as measured by the quinhydrone electrode, but not as measured by the glass electrode.

Under circumstances in which the potential is altered in the presence of interfaces (and even in those in which it is not, as when the amount adsorbed is small with respect to the total amount present) the potential obtained will be solely a measure of the system in the bulk phase, and in no way reflects the reducing intensity at the surface. (It must be understood, of course, that potential or reducing intensity at the surface does not refer to a quantity which can be measured with an electrode, but to a value representing a level of energy which exists whether an electrode is present or not.)

Assuming that the adsorption of the oxidant and of the reductant of a given system is a completely reversible process, describable, e.g., by the Freundlich isotherm, then at each concentration ratio (as long as the bulk concentrations remain below those which saturate the surface) an equilibrium will eventually be set up, involving four components: Red_b, Ox_b, Red_s, and Ox_s (subscripts b and s representing bulk and surface, respectively). Red_b will be in adsorptive equilibrium with Red_s; and Ox_b in equilibrium with Ox_s. Adjustments in equilibria as the concentration ratio, Red_b/Ox_b, is altered from one level to another, keeping total concentration constant, are accomplished not only by adsorption or desorption from the surface, but also by electron exchange among the four components. That such exchange does take place was demonstrated by Abramson and Taylor (1) and by some of my experiments (44), to be reviewed in a later section. The two processes become indistinguishable, and it may appear that oxidative changes in the surface are being accomplished by adsorption and desorption. Thus, if after equilibrium has been reached at a given Red/Ox concentration ratio in the bulk phase, reduction were to be carried out, thereby increasing Red and decreasing Ox, Ox_s may begin to desorb towards equilibrium surface concentration, while part of the reductant, Red_b, may begin to condense on the surface. On the other hand, the equilibrium may be attained by electron exchange, or by a combination of the two, as indicated in Fig. 1. If the adsorption-desorption
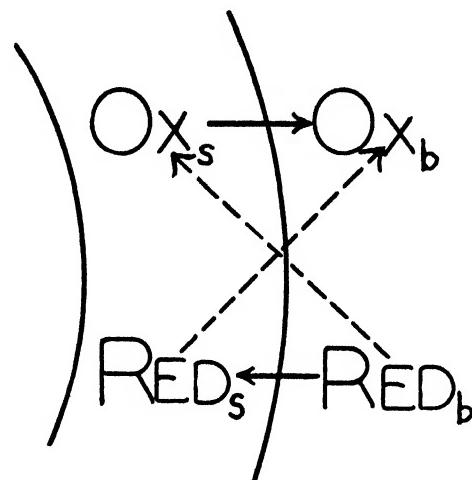
Fig. 1. Ox_s and Red_s represent oxidant and reductant in surface phase; Ox_b and Red_b, same system in bulk (aqueous) phase. Solid arrows indicate direction of movement of Ox and Red (i.e., adsorption and desorption of ions and molecules); broken arrows, direction of electron transfer.

process is relatively slow, most of the adjustment will be accomplished by an exchange of electrons between Red_b and Ox_s and between Red_s and Ox_b. (The electron-exchange process may be more or less separated from the adsorption-desorption process by studying equilibria and oxidation-reductions between a highly adsorbed system and a system which shows no tendency to be adsorbed at the same surface.)

As a consequence of differential adsorbability of oxidant and reductant, it follows, even if pK and pH were to remain unchanged in the surface phase, that the equilibria between two systems will be very different in this phase from the equilibria in the aqueous phase. Thus,

$$\frac{[Red_x][Ox_y]}{[Ox_x][Red_y]} = K_{aqueous} \neq K_{surface}$$

(x, y representing two different systems). $K_{surface}$ will depend upon the adsorption characteristics of each of the four components, the nature of the surface and the shifts in E'_0 due to changes in dissociation and hydrogen-ion concentration.

In addition to being affected by factors controlling ionic dissociation, the potentials, or free-energy changes of oxidative processes at surfaces, must also be profoundly modified by the nature of the bonds between surface and adsorbed molecules of oxidant and reductant. That is, the normal potential (or what corresponds to the normal potential of an adsorbed system) must in some way be the resultant of the potential of the system in aqueous solution and the energies of adsorption of its components. This really is part of the problem of "coordination" compounds (21, 19) and of colloidal "carriers", particularly proteins. As is well known, marked shifts in potential are observed when protein or other nitrogenous substances (-CN, nicotine, pyridine) are added to such systems as hemin (4, 19), phosphoriboflavin (47), and possibly pyridine nucleotides (3). Clark and his co-workers (21) have shown that the direction and magnitude of potential change can be correlated with respective affinities of the nitrogenous compound for the oxidized and reduced forms of the metalloporphyrin. If the reductant forms the more stable compound, the normal potential becomes more positive, and *vice versa*. If the affinities are the same, there is no change in potential. Whether or not a similar analysis can be applied to "coordination" between a surface and a reversible autoxidizable system cannot yet be said; it certainly must be applicable when the nitrogenous compounds form part of the surface, and proteins certainly form a part of most intracellular interfaces. This provides the extremely important possibility (similar to that postulated for the pyridine nucleotides by Barron (3, pp. 212, 213)) that a single intracellular system can act as electron mediator for a great many different oxidations (dehydrogenations) at different potentials, by virtue of its combination with different substances or groups at the activating surface. (Adsorption at inert surfaces, however, involving only van der Waal's forces, must certainly leave the free energy unchanged, but it is difficult to imagine inert surfaces within a living cell.) An investigation of the behavior of well known reversible systems in the presence of a variety of proteins with which they form different kinds of bonds would serve to throw much light on this problem.

In concluding this section, it might be of interest to point out one more consequence of differential adsorption which may be of biological significance. If to an aqueous solution containing two electroactive systems of different potential is added an adsorbent which rapidly and nearly completely removes either the oxidant of the more positive system or the reductant of the more negative system, then the more positive system effectively becomes the more negative system,

since the reductant of the higher system reduces the oxidant of the lower.

Two-Step Systems. This section deals with adsorption phenomena in which constants other than those concerned with ionic dissociation are involved, namely the semiquinone-formation constants of two-step oxidation-reduction systems. This discussion is placed in a separate section because it is not yet certain what the nature of the mechanism is by which these constants are altered —whether it is a matter of hydrogen-ion concentration and dissociation,[2] deformation of the adsorbed molecule, or some other mechanism.

The experiments briefly reported herein bear a strong resemblance to those of Deutsch (24) on pH indicators in that, at the surface, equilibrium is markedly shifted in the direction of a component of the system which in bulk phase appears only in minimal proportions. In these experiments the equilibrium shift is in the direction of increased semiquinone radical formation.

It will be remembered that pyocyanine in solutions more alkaline than about pH 4.9 is blue, becoming colorless, in apparently one step, upon reduction. The intermediate form, present in only very low concentrations, is detectible potentiometrically but not visually. In more acid solutions, the reduction proceeds in two univalent steps, from a red fully oxidized form, to a green intermediate, the semiquinone, to the colorless fully reduced form.

The experiments (44) were essentially as follows. Pyocyanine, dissolved in buffer at pH 5.0, was partially reduced, as in the previous experiments with platinized asbestos and hydrogen, then filtered into the electrode vessels and maintained in the absence of oxygen with a constant stream of nitrogen. A deoxygenated suspension of either kaolin or alumina gel at the same pH was then introduced. After the shifts in potential, if any, were observed, the nitrogen was turned off and the adsorbent permitted to settle. It was frequently found that although the supernatant fluid remained blue, the kaolin or alumina was colored with one of the two-step forms, depending upon the state of reduction. Thus, in some experiments, the adsorbent was light green, while the supernatant fluid was blue; on aeration, the adsorbent eventually became pink, while the supernatant fluid became darker blue. In others, in which the pyocyanine had not been reduced very much, the adsorbent was pink, becoming darker on aeration. In still other experiments, in which the pyocyanine in solution had been reduced to a very light blue, the kaolin or alumina, on settling, appeared color-

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[2] For a given oxidation-reduction system the semiquinone-formation constant may vary to an enormous extent with the pH depending on the ionization constants of each of the components (53).

less. If the aeration and settling were carried out in steps, the adsorbent could be observed to assume, first, the light green color, and then the pink, while the blue supernatant fluid became progressively darker.

Conversely, changes in the opposite direction could be brought about in solutions of pyocyanine, adjusted to just below the turning point (pH 4.80-4.85) with silica gel. In these experiments, the adsorbent became bluish purple while the supernatant fluid remained pink or red. On the alkaline side, however, both solution and silica remained blue.

In that the pH of the bulk phase in these experiments was so close to the turning point of the dyestuff, these experiments are not very satisfactory, but they at least demonstrate the definite possibility that at intracellular interfaces univalent oxidations may predominate despite the unfavorableness of the protoplasmic reaction. This was more strikingly demonstrated by Haas (34) who showed that the yellow enzyme, when reduced by triphosphopyridine nucleotide (coenzyme), in a nearly neutral solution, passes through a red intermediate state. This red component is apparently identical with the radical obtained with the free lactoflavin only in extremely acid solution (pH < 0). In other words, as pointed out by Michaelis and Schwarzenbach (52), the semiquinone radical formation constant is greater for the flavin-enzyme than for the free lactoflavin, and the electronic structure of the radical in combination with the protein and the coenzyme resembles the structure of the free radical in extremely acid solution, where it is in combination with a proton. The combination with a specific protein and a coenzyme displaced the equilibrium in favor of the radical, and this amounts to an activation, or a step-up in free energy.

Changes such as these at interfaces deserve considerable emphasis and attention. Their broad significance becomes especially clear in the light of the fundamental role of semiquinone radicals in biological oxidations, as expressed in Shaffer's (66) principle of valence harmony, and Michaelis' (53) principle of compulsory univalent oxidation, which embodies the former (3, pp. 210, 211).

Little can be said at the present time as to how the increased semiquinone formation is brought about at the surface. It appears significant that in the experiments just reported, the change could be brought about only at solid, ionogenic surfaces, presumably involving high energies of adsorption. Attempts to demonstrate similar effects with cellulose or in emulsions of benzene, toluene, chloroform, etc., (as in Deutsch's (24) experiments) were completely negative. Of importance, also, is the fact that the adsorbents were metallic compounds. This brings to mind the experiments of

Fajans (28) in which color changes in certain dyestuffs were induced at the surface of insoluble metallic compounds, and which were attributed by Fajans to distortion of the molecule. Certainly the bonds are stronger than those of ordinary adsorption.

Kinetics of Oxidative Reactions in Heterogeneous Systems. The problem of reaction velocity is, of course, not dissociable from that of equilibrium. It has already been shown that chemical equilibria of many kinds can be drastically altered at surfaces. It is no exaggeration to say that selective adsorption of the various constituents of a solution may be a controlling factor in a great variety of natural processes, for the power of regulating concentrations is associated with the power of influencing the rate of chemical reactions.

It is found nearly invariably that in the presence of interfaces, even those of mild adsorbents, kinetics of a great variety of reactions are considerably altered, and in most cases reaction velocities are reduced. Those who have observed the retardation of reactions at surfaces often express surprise. This perhaps arises from the habit of thinking of intracellular surfaces as promoting reactions rather than slowing them. This habit is based, I believe, upon two assumptions: first, that whereas, in solutions, velocity of reaction is determined by probability of collision of the reacting components, in the cell, randomness is minimized by oriented surfaces; second, that mere increase in concentrations at surfaces, by adsorption, will serve to accelerate reactions according to the mass law. These factors and others will be considered in the following discussion.

It is unfortunate that very few studies on the kinetics of oxidation-reduction in heterogeneous systems are available. It will be necessary, therefore, to draw upon other types of reactions for examples. Freundlich (31) has shown that charcoal strikingly alters the kinetics and equilibria of the following reactions:

(1) $CH_2BrCH_2NH_2 + NaOH \rightarrow$
$$\begin{array}{c} CH_2 \\ CH_2 \end{array} > NH + NaBr + H_2O.$$

(2) $\begin{array}{c} CH_2 \\ CH_2 \end{array} > NH + HBr \rightarrow CH_2BrCH_2NH_2.$
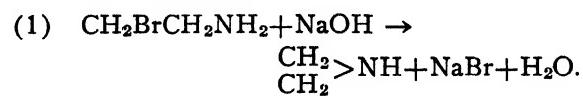
Freundlich conducted his experiments in such a fashion that the bromethylamine, which is much more highly surface active than the dimethylenediamine, was completely adsorbed on the charcoal. He found, as a consequence of this differential adsorption, that reaction 1 was retarded while reaction 2 was accelerated, with consequent alteration of the equilibrium constant. Not only were the reaction velocities altered, but the mechanism

of the two reactions in the presence of charcoal was distinctly different, as indicated by changes in the kinetic order.

The question now arises, why should a reaction be slowed by the mere presence of adsorbing interfaces? The decrease in the aqueous phase is ascribable, of course, to the lowered concentrations caused by adsorption. The net decrease, however, indicates that the reaction must be proceeding very slowly, if at all, at the surface. We should expect, if some form of the mass law operates at surfaces, that the mere increase in concentration at the surface would accelerate the reaction. But the decrease in reaction rate appears in so many reactions that it is worthwhile seeking further for the cause of the retardation.

In the first place, can intrinsic reactivity itself be modified by reason of mere presence at a surface? (Specific catalysts and enzymes at which reactivity is greatly modified as a result of intramolecular changes or complex-formation will not be considered here.) In general, it appears from evidence from the studies of Rideal, Hughes, and others, on monomolecular films (2), that the mere presence of molecules at a liquid surface does not change their intrinsic reactivity, as measured by the energy of activation.

A decrease in reactivity, perhaps even to zero, may be expected, however, if the bonds responsible for adsorption involve those groups responsible for the reaction. Abramson and Taylor (1) investigated this possibility for oxidation-reduction processes. They found that methylene blue, phenosafranine and litmus remained capable of being repeatedly reduced (with hydrosulfite, thiourea or cysteine) and reoxidized (with oxygen, quinone or ferricyanide) while adsorbed on filter paper and other surfaces. They concluded that the adsorption bonds do not appear to affect the groups involved in the oxidation-reduction process.[3]

Factors concerned with molecular mobility and molecular orientation have received emphasis from Kruyt (46). In 1916 he had been struck by the non-operation of the mass law at interfaces. He had found that the presence of interfaces (charcoal) invariably slowed the reactions which he was studying, e.g., the saponification of ethyl acetate by NaOH. The greater the adsorption, the slower the reaction. On the basis of the belief that the investigation of substances which are at once highly water soluble and strongly adsorbed might yield some clue, he turned, in 1919, to hydrolyses and substitution reactions by halogens in

certain derivatives of cinnamic acid. The following are some of the reactions which he studied, in homogeneous solution and in the presence of charcoal.

$$p\text{-}NaO_3S\text{---}C_6H_4\text{---}COOCH_3 + NaOH$$
$$\rightarrow NaO_3S\text{---}C_6H_4COONa + CH_3OH$$

$$p\text{-}NaO_3S\text{---}C_6H_4\text{---}CH\text{=}CH\text{---}COOH + Br_2$$
$$\rightarrow NaO_3S\text{---}C_6H_4\text{---}CHBr\text{---}COOH$$

$$p\text{-}NaO_3S\text{---}C_6H_4\text{---}CHBr\text{---}CHBr\text{---}COOH + 2KI$$
$$\rightarrow NaO_3S\text{---}C_6H_4\text{---}CH\text{=}CH\text{---}COOH\ 2KBr + I_2$$
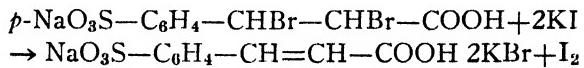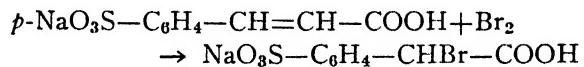
These reactions, too, were strongly retarded by charcoal. He found these phenomena interpretable on the basis of orientation of these molecules at the surface, and, therefore, on the basis of the accessibility of the reacting groups. Since the sulphonate group is the most polar in the molecule, it will be attracted to the water away from the surface, leaving the other end of the molecule, which bears the reacting group, more or less buried in the adsorbed layer, and therefore not readily accessible to the dissolved molecules of NaOH, Br₂, or KI.

The importance of orientation was further emphasized in later experiments with similar reactions of organic compounds in which the reacting groups were placed in the most polar end of the molecule, or so placed that they were brought into very favorable positions on the surface for reaction. Retardation, however, was observed even with favorable orientation. (Acceleration occurred only when some form of activation took place, as when the reacting molecule itself underwent "distortion" or "stretching" on the surface, in such a way as to render its reacting groups even more accessible than in the dissolved molecules of the same compound.)

It is true that the retardation is much less when the orientation is favorable, but there is still no acceleration. However, as Kruyt points out, the slowing of a reaction by unfavorable orientation at a surface gives no a priori grounds for assuming that a reaction will be accelerated by favorable adsorption. Retardation, even with favorable orientation, is explainable, according to Kruyt, on the basis that first, adsorption involves decreased mobility and therefore lowered probability of collision, and secondly, this probability is not increased, in comparison to homogeneous solutions, when favorable orientation takes place.

The importance of orientation and accessibility of molecules and their reacting groups, in the kinetics of reactions at interfaces, is further well demonstrated in certain recent work on monolayers spread on liquid surfaces (Schulman, Rideal, Hughes, Harkins, Langmuir and others)

[3] I believe that this conclusion can as yet be only provisionally accepted, in view of the fact that the rates of oxidation and reduction were conspicuously diminished. For instance, the adsorbed leuco-dyes were much more slowly oxidized in air, even after thorough washing, than were dissolved leuco-dyes, and the oxidants appeared more resistant to reduction.

which will not be reviewed here, except in principle. The accessibility of reacting groups in the film to the molecules and ions in the underlying solution with which they react, can be controlled by changes in cohesion, changes in compression due to changes in pressure on the barrier, or to other mechanical factors, and by changes in composition of the underlying solution, as in pH, electrolyte content, heavy-metal ions, etc. This amounts to control of the steric factor, and consequently, the rate of reaction of a substance (and apparent energy of activation) in a film may be greatly influenced by the structure of the film. Quite a variety of reactions, including oxidation-reductions, hydrolyses, polymerizations, and enzyme reactions, have been studied; rates of reaction are ordinarily followed by changes in surface pressure and surface potential.

Fortunately, at least one good example from the field of oxidation-reduction is available for illustration of these principles. Hughes and Rideal (39) have observed striking changes in rates of oxidation of oleic and petroselinic acids, spread in monofilms over dilute acid permanganate, when the degree of compression of the films is varied. The rate of oxidation may be reduced ten-fold by crowding the molecules in the film. The reasons for this phenomenon become clear from a consideration of the following facts. Both acids have a double bond in the middle of the chain and form liquid-expanded films; on contact of the double bonds with the underlying permanganate they become oxidized, taking on hydroxyl groups which, being strongly hydrophilic, cause the molecule to lie flat (gaseous film). The probability of contact between the double bond and underlying solution is much greater when the molecules in the film are permitted to tilt freely, than when they are held upright or nearly upright by compression.

In view of the fact that the relation between orientation at interfaces, or structure of adsorbed layers, and rates of reaction provides a mechanism whereby the cell can control reactions by influencing the accessibility of reacting groups to the reagents in the bulk of the cell, further studies of this nature, on biological systems, would be of considerable interest.

Another factor which must be taken into consideration in the study of oxidation-reductions in heterogeneous systems is that of diffusion. In fact, Nernst's general theory of the kinetics of heterogeneous reactions is largely concerned with this factor, and based upon such studies as those of Noyes and Whitney in which the rates of dissolution of solid substances in water were measured. In reactions between dissolved substances and those existing at surfaces, or between dissolved substances and solids, there are three distinct processes involved (54) : (1) The solute molecules approach and collide with the solid surface, where (2) the chemical reaction proper takes place, followed by (3) the recession of the products away from the interface into the bulk of the solution. Theoretically, each of these three processes, having definite velocities, may influence the net rate of reaction, the magnitude of which is determined by the slowest process of all. Since (1) is usually faster than the chemical reaction itself, the determining factor often will be the relative magnitudes of the rate of the chemical reaction itself and of the rate of diffusion of the resultants from the interface. Diffusion, therefore, becomes a very important and possibly a controlling factor in heterogeneous reactions. In many cases, the observed velocity is simply the velocity of diffusion, and the true kinetics of chemical change are obscured.

It is very improbable, however, that reactions at the surfaces of submicroscopic particles are diffusion-limited. Such particles, being in rapid Brownian movement, are not surrounded in the same manner as larger particles or bulk material by a constant diffusion layer (13a). If the Brownian movement of such particles be eliminated by fixation on larger bodies or on some framework, e.g., within the living cell (see last section), then, of course, reactions at their surfaces will show the influence of diffusion rates.

Thus far, most of the factors considered have contributed to the retardation of reactions in heterogeneous systems, as compared with homogeneous reactions. An important factor (aside from specific catalysis) through which adsorption at interfaces may accelerate reactions has been pointed out by Born and his co-workers (11), a full theoretical discussion of which is beyond the scope of this paper. According to the Arrhenius concept, at any moment only a fraction of the molecules are in a reactive state, and require a certain energy of activation to bring them into this state. The increased energy may be that of translational or vibrational motion. In considering activation for chemical reaction, the vibrational energy has recently received much attention, particularly in connection with what is called the "transition" state (see 27). Since the time of contact between two colliding molecules (especially in gases) is extremely brief, the probability of the transition or reactive state being reached in both molecules during time of contact is extremely small. When, however, the two molecules are adsorbed side by side they may be held for periods very much longer, so that the chance of vibrations passing through the transition state is very much increased. It is now said that the chance of reaching a reactive state in two molecules simultaneously increases with the square of the time during which they are in contact, so that the function of the surface is to hold them close together long

enough "for them to make up their minds to react" (2).

In experiments with adsorbents I (44) have found that invariably adsorption of dyes at interfaces (cellulose, kaolin, silica or alumina) slows the reduction of the oxidant and oxidation of the leuco-form. When oxygen is passed through a suspension of adsorbent in a solution of leuco- or partially reduced dye, the appearance of color in the adsorbed layer is much slower than in the buffer solution and the adsorbent may remain uncolored even after the dissolved dye has recovered most of its color. In one experiment, for instance, at pH 2.36, with kaolin and pyocyanine, in which the solution was light green (between the first and second steps), the kaolin was found to be almost colorless on settling out after the experiment. On bubbling air through the suspension, the solution gradually turned pink (fully oxidized form). On settling out again, the kaolin was observed to be green (intermediate form). After repeated bubbling and settling, the adsorbed pyocyanine remained predominantly in the intermediate form long after complete oxidation of the dissolved dye. Conversely, as in experiments to be described in the next section, when dissolved dyestuffs in contact with the same dyestuffs on adsorbents are reduced, the decoloration of the adsorbed dyes lags far behind that of the dissolved dye, and, indeed, may remain colored for several hours after the other has become completely colorless.

Whether these reduced rates of reaction are due to the diffusion factors previously outlined, or to factors of orientation, mobility, intrinsic reactivity or to formation of new compounds, *e.g.,* silicates (64), cannot yet be determined.

It is clear that in all cases where the oxidation-reduction of the system at the surface lags behind that in the aqueous phase, the oxidation-reduction potential of the homogeneous phase, either as indicated by color intensity or by electrode, is by no means indicative of the reducing intensity of the surface system. It is not unlikely that these rate factors may in large part be responsible for situations frequently reported for cells, in which intracellular pigments are found in a state of reduction apparently incompatible with the intracellular reducing intensity (indicated by injected indicators). The earliest case reported is that of hermidin, estimated by Cannan (14) to be present in the cells of *Mercurialis perennis* 95 p.c. reduced (theoretically in equilibrium with a pressure of oxygen of only about 10^{-60} atmospheres) despite the fact that oxygen is being produced within the same cell by the chloroplasts.

Another case is that of the sea urchin egg, in which the echinochrome granules show no apparent fading when the egg is subjected to anaerobiosis, despite the known increase in reducing intensity. Many other similar cases have been reported in which the intracellular pigments, occurring in granules or vacuoles, are in a state of oxidation-reduction at variance with the intracellular rH. Although, as suggested above, these apparent differences in potential, *e.g.,* between the hermidin and the protoplasmic systems, may be maintained in a steady state by differences in rate of oxidation-reduction, it must be remembered that the potentials of the intracellular pigments are estimated on the basis of potentiometric studies of the extracted form in homogeneous buffer solution. It is not unlikely that these estimated potentials, at least in some cases, greatly exaggerate the inconsistency, in that it has not been taken into consideration that potentials are greatly altered by the presence of the pigment at interfaces, and by the presence of proteins with which the pigment may combine.

Effects of Localization of Reactions on Separate Surfaces. One interesting corollary of a polyphasic medium for oxidation-reduction processes is that two substances which ordinarily react very readily in homogeneous systems, may show no tendency to react in the presence of interfaces. This condition arises when the two systems are separately adsorbed on different surfaces. There is no appreciable reaction even when the two surfaces are in very close contact.

This principle was demonstrated by the ingenious experiments of Kautsky and Baumeister (40). They prepared two catalyst-adsorbent systems; one consisted of $Pt(NH_3)_4^{++}$ adsorbed on negative silicic acid, the other of $Pt(OH)_6^{--}$ on positive $Th(OH)_4$ gel. Methylene blue, the indicator dye which they used, is adsorbed by silicic acid but not by thorium hydroxide. The rate of reduction of the dye was indicated by the rate of hydrogen consumption observed manometrically. They found that hydrogenation (reduction) is retarded by polar adsorption of the dye, the retardation increasing as the concentration of the dye in equilibrium with the adsorbed dye is decreased. If the Pt and dye are adsorbed on separate surfaces (Pt on $Th(OH)_4$ and Mb on silicic acid) the same curve is obtained as if both were on the silicic acid. In all cases the rate of reduction was determined by the equilibrium concentration in the aqueous phase, and not by that on the adsorbing surface. As long as the surface of the Pt itself is saturated, the rate of reduction is constant. The results indicate that hydrogenation takes place only in molecules that reach this surface from the solution. Within the boundary surface itself it appears that the dye molecules adsorbed by the gel are incapable of diffusion, and therefore do not reach the actively catalytic surface of Pt particles;

hence hydrogenation of these molecules does not take place.

Michaelis, and Abramson and Taylor (1) also found that if filter paper, stained in methylene blue and thoroughly washed, is suspended in a stream of hydrogen in water or buffer solution, in the presence of platinized asbestos, reduction of the adsorbed methylene blue does not occur to any appreciable extent. I confirmed this (44) with filter paper, pure cellulose and silica gel and several dyestuffs. The explanation in these cases is similar, of course, to that given by Kautsky and Baumeister for their results. The hydrogen is active only at the surface of the Pt particles and, therefore, can hydrogenate only those dye molecules which are adsorbed on the same surface. If the dye is on another surface there is no reaction.

Just as two systems in separate vessels can be made to interact ("reaction at a distance") by providing a means for conduction of electrons (by connecting them with metal and a salt bridge), so can the two systems on separate surfaces. If instead of suspending the stained adsorbent and platinized asbestos in plain buffer solution, one also adds a little dissolved dye, then it is found that as the dissolved dye fades the adsorbed dye also begins to become reduced. In other words, the dissolved dye acts as electron carrier between the two surfaces.[4] The reduction of the adsorbed dye lags far behind that of the dissolved dye; the lower the concentration in solution, the slower the reduction of the adsorbed dye. Similar phenomena are observed if the dissolved dye is not the same as the adsorbed one and is not adsorbed.

Because of these localization phenomena, it is not justified to conclude, on the basis of observations that two substances which, when they are extracted from cells, show great tendency to react, also bear some intimate functional relationship to each other within the cell. Certain inconsistencies, such as some of those observed by Ogston and Green (58) between reactions of certain systems *in vitro* and reactions of the same systems *in vivo*, may be based upon such factors.

Electron Conduction at Interfaces. This section is concerned with a question which has been raised repeatedly—but rarely more than raised—in connection with biological oxidation-reductions, coupled oxidations in cells, energy transfer, etc. It is a question which, even with our present meagre knowledge, is not impossible of investigation, and in view of its fundamental significance certainly deserves more attention than it has received. The question, stated in its simplest form is this: Can effective electron-transfer be accomplished along or across (non-metallic) interfaces, films or membranes? The same question is often implied in others: Can oxidation-reduction potentials be a source of bioelectric potentials? Are diffusible mediators (hydrogen-carriers) necessary for the electron transfer between dehydrogenase-substrate systems or can connecting surfaces serve the same function?

It appears that the question was raised originally (from the biological point of view) in connection with bioelectric potentials, as for instance in the investigations of Lund and his co-workers (50), who have postulated that oxidation-reductions can be the direct source of potentials in cells. The objection most frequently urged against the oxidation-reduction theory of bioelectric phenomena is that metallic electrodes are absent from the cell, and non-metallic electrodes, which cannot be used to measure oxidation-reduction potentials, are used for the measurement of bioelectric potentials.[5] This argument, as pointed out by Marsh (51), is entirely *a priori*. A metal electrode is not essential to the formulation of equations expressing the potential of an electrochemical system, nor to its manifestation. "Metallic" conduction is not restricted to metals, and normal cell structures possessing the essential properties of an electrode are physically possible. Carbon chains set side by side at a surface in oriented fashion are thought to be capable of electron conduction (*cf.* graphite); other mechanisms will be discussed later.

The existence of such structures or interfaces would make it possible for oxidation-reduction potentials to be measured by non-metallic electrodes, *e.g.*, calomel electrodes, which themselves are incapable of filtering electrons. Fig. 2 indicates an arrangement in which such a condition exists. Two solutions of reversible electroactive systems are separated by a noble metal "membrane"; the salt bridge of a calomel cell is immersed in each; the two are connected through a potentiometer. The E.M.F. in each case will be equal to the differences in E_h of the two solutions. If one side is maintained fixed at a given percentage reduction while the other is varied, *e.g.*, by the introduction of substrate-dehydrogenase systems or of washed bacteria plus substrates, then the electrode potentials become directly related to the "metabolic" activity. Or if to the variable side, containing only the electroactive system and a dehydrogenase, is suddenly added a substrate specific to the dehydrogenase, there will be an abrupt change in potential, the direction depending upon whether the oxidant or the reduc-

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[4] It has been pointed out that this mechanism may be important in the transfer of energy from surfaces at which energy-releasing oxidation-reductions are taking place to those at which energy is needed, *e.g.*, syntheses (44).

[5] Beutner (6), particularly, has raised this objection; he has, himself, however, produced evidence that potentials at phase boundaries, as measured by non-metallic electrodes, may bear direct quantitative relationships to certain organic oxidations. I have adduced other evidence in unpublished experiments.
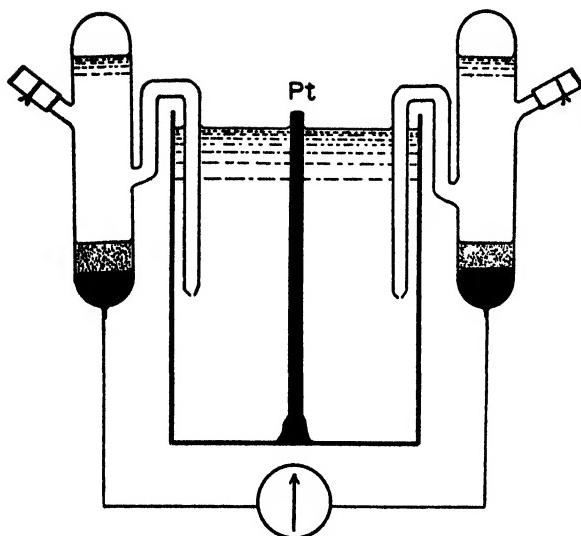
FIGURE 2.

ant of the substrate-dehydrogenase system is added, the magnitude depending upon the amount added.

The close correlations between the after-potentials in nerve, as to magnitude, course and direction, and the oxidative metabolism, seem to indicate that the relation is a rather direct one, as contrasted with one in which a membrane is dependent upon the energy released by oxidations only for its maintenance and polarization. It is not impossible that the potentials are directly determined by, and related to, the percentage oxidation of some system (the concentration of which increases during activity), e.g., lactate-pyruvate.

Lillie (48), too, seems convinced that there is something in the surface of nerve fibers, analogous in function to a metal, and at which oxidation-reductions occur in dependence on a flow of current.

Direct electron conduction has also been accepted as a distinct probability in biological oxidations by Borsook (12), who has called attention to the hydrogen-bond as providing such a mechanism. Direct electron conduction has its place, he believes, in the so-called coupled oxidations of the type:

(1)   pyruvate$^-$+formate$^-$+H$_2$O $\rightleftharpoons$
                              lactate$^-$+HCO$_3$$^-$

and

(2)   fumarate$^-$+lactate$^-$ $\rightleftharpoons$
                       succinate$^-$+pyruvate$^-$.
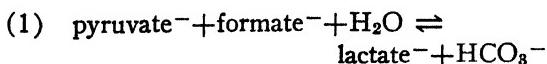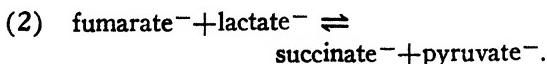
In the presence of toluene-treated bacteria these reactions do not take place unless a dye of the proper potential is added; that is, some inter-

mediary mechanism is required for carrying electrons from the locus where the formate (reaction (1)) is oxidized to the locus where pyruvate can be combined with hydrogen ions and the electrons, and so converted to lactate. (Compare the experiment with the methylene blue-dyed filter paper and colloidal platinum "mediated" by dissolved dye.) In the intact bacteria no intermediary dye needs to be added. Borsook has concluded that in the untreated organism direct electron transfer takes place between the dehydrogenase "complexes" and that the mechanism for this conduction is destroyed by toluene, by virtue of the surface activity of toluene and its affinity for lipoids.

Although Borsook's evidence may not appear very convincing, I feel, nevertheless, that surfaces which are capable of such phenomena as the activation of molecular hydrogen (32) must also have other properties in common with noble metal surfaces.

Thus far only two suggestions have been made with respect to the mechanism of electron conduction at surfaces—oriented layers of carbon chains and the hydrogen bond. Recently evidence for "metallic" conduction across non-metallic membranes has been accumulating from the study of electrostenolysis.

In 1867 Becquerel (5) carried out some extremely interesting and important experiments with test tubes which had received very fine cracks. If such a tube was filled with a solution of Cu(NO$_3$)$_2$ and immersed in a solution of Na$_2$S, at first CuS, and then crystalline Cu, deposited on the side of the cracks facing the Cu(NO$_3$)$_2$, and on the other side a yellow liquid which contained Na-polysulfide spread into the solution. Since the solutions on the opposite sides of the tube were strongly oxidizing (Cu(NO$_3$)$_2$) and reducing (Na$_2$S), a potential difference was set up wherever they made contact, producing local currents which induced the chemical changes.

This old observation has been extended by many observers to a variety of other systems (inorganic salts), using many different kinds of barriers, including extremely fine capillaries, narrow tubes packed with finely ground glass, silica, sulfur (38) and membranes made of a great variety of substances, e.g., water glass, lecithin-collodion, rubber and cellulose acetate (49, 30), potentials being applied across the barriers from an external source. In the past workers have been interested in the formation of metallic deposits on the barrier surface when a potential is applied across it. More recently this phenomenon of electrostenolysis has been extended by Fetcher et al (30; see (29) for further theoretical and historical treatment) to include the oxidation-reduction of organic substances in which no metallic deposits

are involved, and has been re-defined as follows: The phenomenon of electrostenolysis consists of the oxidation and reduction of a solution at the opposite surfaces of a high-resistance membrane which separates the two electrodes of an electrolytic cell. Reduction of the solution takes place at the surface of the membrane facing the anode, oxidation at the surface facing the cathode, and the process occurs only if there is a steep potential gradient across the membrane. Some of the effects of the membrane are the same as those of a metallic conductor in the same position in so far as ions are oxidized and reduced at the surfaces of both, *i.e.* electrons are transferred.

Fetcher (30) devised an apparatus for the quantitative study of electrostenolytic oxidation-reduction of the Fe^{++}-Fe^{+++} and methylene blue-leucomethylene blue systems, using cellulose acetate as a membrane. In confirmation of older workers (49), it was found that a minimum potential gradient is required before the process starts; for the ferrous-ferric system and a cellulose acetate membrane, about 2150 v./cm., must be attained in order to effect any oxidation and reduction. The corresponding figure for methylene blue·is apparently higher. Since the membranes are very small fractions of a centimeter (of the order of 5×10^{-4} cm.) the actual potentials were, of course, much smaller. One important difference between the electrostenolytic membrane and an electrode is that the ratio of Faradays passed to equivalents oxidized is not unity, but is typically of the order of several thousand to one. However, the similarities between the electrostenolytic membrane and a metal are so marked, and as Lillie (48) has pointed out, electrostenolysis bears such strong resemblances to certain vital membrane phenomena (*e.g.*, some of those concerned with nerve excitation) that the phenomenon must definitely be considered as a mechanism of electron transfer in cells, especially in view of the existence in cells of high-resistance, highly polarizable membranes and films, and of the steep potential gradients across them. The existence of a minimum potential is reminiscent not only of excitation phenomena, but also of oxidation-reduction systems adsorbed on intracellular interfaces or in separate phases at apparent potentials very different from those of the continuous phase, as if certain potential differences must be exceeded before electron exchange takes place.

One other mechanism of electron conduction by non-metallic structures is only just beginning to attract attention, and has been applied in the study of relations between cellular oxidation and secretion (69). A reversible electroactive system, such as methylene blue, "fixed" in a high-resistance membrane, must render that membrane electron-conducting. Such membranes, placed in the position of the metal partition of Fig. 2, are now under investigation and will be the subject of a later report.

One cannot stress too strongly the urgent need, in biochemistry and biophysics, for a fuller understanding of phenomena of this nature.

Oxidations and Cell Structure

As indicated in the introduction, it is not enough to think of cellular oxidations as taking place merely in a colloidal medium, considering only the modifications imposed on substances and their reactions by intracellular interfaces. A much higher order of complexity and organization is certainly involved. The integration of the multitude of oxidative and other reactions into cellular metabolism, and the coordination of metabolism with other vital processes must in large part be due to a high degree of cellular organization. It is important to inquire how far the control of cellular metabolism and its individual processes is due to cellular organization; how far chemical and physical states of intracellular substances and equilibria are determined by this organization and its changes; and what new phenomena and properties arise from this organization.

One need only briefly consider some of the effects of disruption of cellular organization to appreciate its significance in cellular metabolism. In most cases when cell structure is destroyed, as by cytolysis or by mechanical means, oxygen consumption is considerably reduced; in others it is increased, due either to the fact that many oxidations go to completion, which in the cell are halted at specific stages, to the interaction of molecules which in the cell are prevented from meeting, or to the "activation" or release of catalytic systems which in the cell are regulated at a low functional level. For instance, the respiration of the sea urchin egg is reduced to about 10 or 12 p.c. of the original upon cytolysis (72), whereas amphibian gastrulae take up five times as much oxygen after cytolysis as before (13). In many cases specific oxidations are lost, *e.g.*, liver slices when minced lose the capacity to oxidize fatty acids; the luminescent reaction in bacteria is also dependent on cellular integrity (42). The capacity for glycolysis is destroyed upon hemolysis of mammalian erythrocytes; plants become discolored upon injury; transfer of energy from oxidations to syntheses is interrupted upon grinding tissues.

These phenomena are further demonstrated in extracts and "reconstructed" systems which show very many fundamental differences from the original systems. Substances which ordinarily are only transitory (*i.e.*, represent brief steps in a chain of enzymatic reactions) may be stabilized and accumulated even though the enzymes which in the cell are responsible for their removal and

conversion are present, e.g., certain phosphate esters in muscle, or other carbohydrate breakdown products (cf. 15). Substances and systems which in the cell apparently do not interact may do so in extracts, or vice versa. Important quantitative changes also occur, of course. For example, in living yeast, the frequency of oxidation-reduction of cytochrome is about 4000 per minute (71), whereas in extracts, under optimal conditions, only about one-tenth of this value is obtainable (58). Further interesting differences have been described by Ogston and Green (58) in their studies on the function of various mediators in reconstructed systems and of the same systems *in vivo*. *In vitro*, for instance, only succinate in tissues, and lactate in yeast, showed any appreciable tendency to react with oxygen through cytochrome, whereas the evidence is overwhelming that a large variety of substrates are dependent upon the ferrous systems, and that, indeed, the Warburg-Keilin system may, in certain cases, be responsible for nearly the entire oxygen uptake (51, 22).

Not only are the relations between catalytic systems upset by cell destruction, but in many cases the enzymes themselves are dependent on and associated with microscopic structure. Perhaps nowhere is this more clearly demonstrated than in the bacteria, in which the dehydrogenases seem to form a part of the cell wall (63, 59). Cytochrome oxidase and the oxidase of phosphopyridine nucleotides are also known to adhere very closely to the solid structures of the cell.

Studies on damaged and destroyed cells, important as they are, reveal little of the nature of cellular organization and its relation to metabolism; they point only to its importance. One must turn to normal, intact cells, to the alterations in cellular metabolism that result from changes in organization accompanying certain natural processes (e.g., embryonic development, stimulation), or that result from certain experimental means.

The centrifuge, for instance, which has so long been used in experimental cytology and embryology for altering the organization and distribution of intracellular material (for review, see 37), has found fruitful application in the field of cellular metabolism. The interesting experiments of Shapiro (67) on the respiration of fragments of sea urchin eggs may be taken for example. When the egg was split into a light half and heavy half (each of which is capable of continued development) by centrifuging, the respiration of the heavy half was about 2.2 times as great as that of the light half, but only the light half showed an increase in respiration upon fertilization. In other words, the factors responsible for the differences between the two halves, and for the increase in respiration that normally occurs on fertilization

must be associated with particles which are easily moved by gravitational forces. These particles are normally part of an organization which becomes restored to normal after centrifuging (if the egg has not been fragmented), as shown by embryological data.

A similar principle is demonstrated in the experiments of Bodine and Boell (8). Pre- and post-diapause (actively developing) grasshopper embryos which, like fertilized sea urchin eggs, are cyanide-sensitive, showed a considerable reduction in oxygen consumption after ultracentrifuging. It seems reasonable to assume that by centrifuging, mutually interdependent systems, held in proper relation to each other by normal cell structure, have been separated. I (43) have previously attributed this effect to the segregation of cytochrome oxidase (i.e., of the particles or formed bodies with which it is associated) to one end of the developing egg, which deprives many of the dehydrogenases of their hydrogen outlet. On the other hand, the diapause (or "blocked") embryos which, like the unfertilized sea urchin egg, are cyanide-insensitive, are unaffected by forces of even 400,000 times gravity. The dehydrogenases, during this stage, very likely depend upon one or more soluble, diffusible hydrogen-carriers which cannot be moved by centrifuging, and which remain uniformly distributed and in contact with the proper enzyme surfaces. It is no coincidence, I believe, that the Warburg-Keilin system, which is one of the most variable respiratory systems in the cell (see later), seems also to be especially closely bound up with, and affected by, alterations in cell structure.

Fixed and reproducible spatial relationships among enzymes are implied in many features of normal cellular respiration. Serial dehydrogenation, in which a given foodstuff undergoes a series of dehydrogenations (or dehydrogenations and hydrolyses), the product of one becoming the reactant of the next, very conceivably has its ultimate basis in a specific arrangement of the enzymes, maintained by some microstructure in the cell. Contiguity or physical concatenation of enzymes which cooperate in oxidative or hydrolytic breakdown has been emphasized many times (15, 55, 62). It has been suggested that in many cases the effects of elevated temperatures or of certain electrolytes are not upon the enzymes themselves, but upon the colloidal links between them. The accumulation, in extracts or *brei*, of substances which normally are transitory, is probably accounted for by the interruption of these enzyme chains.

Perhaps the most fruitful approach to the problem of the relations between micromorphology and cellular metabolism is provided by the systematic study of the chemistry of embryonic de-

velopment. It has been repeatedly demonstrated in recent studies on early stages that not only is there an energetic link between metabolism and the embryonic processes, but also a more material one, through which metabolism itself becomes markedly altered as a result of cellular reorganization and differentiation. One must view it as a reciprocal relationship in which the rates, mechanisms and interrelations of chemical processes and their catalysts become revised in reflection of the morphological changes for which the chemical processes provide the necessary energy. The classical illustration of this relationship is the sea urchin egg. The unfertilized egg has a low respiration which is unaffected by cyanide. Immediately upon fertilization, or upon artificial activation with agents which can produce the requisite changes at the surface of the cell, there is a marked increase in respiration involving, among other changes, the introduction of a large cyanide-sensitive fraction. (For literature and fuller discussion, see 43.) Although the Warburg-Keilin system is present in the unfertilized egg, and capable of functioning as rapidly as it does in the fertilized egg, as shown by specific tests, it becomes geared to the rest of the respiratory system only as a result of the abrupt structural and chemical reorganization which is initiated by certain changes at the surface of the egg, and which sets in motion the developmental machinery. Inhibition of the Warburg-Keilin system also stops development; restoration of the original rate of respiration of the fertilized egg with added carriers does not restore development; this can be accomplished only by removing the inhibitor. It seems, therefore, that the energy released in the "basal" metabolism of the egg is insufficient for, or inadequately transferred to, the embryonic process, and that the additional energy released by the increased respiration of the fertilized egg requires the activity of the ferrous systems in order that it be properly transferred to the embryonic processes. That is, this reorganization, in setting off the growth and differentiation processes also taps and links to these mechanisms new sources of the requisite energy. It is difficult to visualize the mechanism of such integration except in terms of a specific and reproducible cellular architecture, of which the cell's catalysts form a part.

A parallel situation, as indicated above, exists in the grasshopper embryo (7). During its development it passes through a period of quiescence, known as the diapause, during which growth and differentiation are suspended. The diapause egg has a low respiration which is HCN- and CO-stable. The pre- or post-diapause egg, however, has a much higher respiration, the difference being equal to that proceeding through the cytochrome-cytochrome oxidase system and in-

hibitable by cyanide and monoxide. In other words, the accessibility of the Warburg-Keilin system is governed by developmental changes, and when it is inaccessible, growth and differentiation appear to be impossible.

Only two examples have been presented here, but the embryological literature is rich with illustrations of the extremely close relations between a great variety of metabolic processes on one hand and growth, differentiation and morphogenesis on the other. All point to the fundamental significance of chemical geography and structural relationships in cell metabolism. The morphogenetic hormones (organizers, inducers, evocators) whose effects appear as changes in cell and tissue architecture (presumably through re-orientations of intracellular protein molecules) must certainly have their metabolic origins and relationships. The morphological changes which they induce are further reflected in metabolic changes. These studies (such as those of Brachet, Needham, Runnstrom and others) cannot be reviewed here, but are summarized in various reviews (56), and in the recent series of papers by Boell, Needham, Koch and Rogers (10).

It is of interest to point out in this connection that Stier and Newton (70) have concluded that the respiratory changes that occur in yeast at the beginning of anabolic activity are directly or indirectly due to a reorganization of the "finer cytological structure of the cell".

Pointing in the same direction are the abrupt, reversible, unified and integrated changes in metabolism which accompany the specialized responses of cells and tissues to external stimuli and other changes in the functional activity of cells. The chemical changes accompanying and following muscular contraction and axon conduction are becoming well known. It is important, however, to emphasize as well less familiar systems, e.g., those concerned with secretion. Deutsch and Raper (25) have confirmed, with refined techniques, older observations on the increased respiratory rate when mammalian salivary glands and pancreas are stimulated to secrete by their specific humoral agents (acetylcholine and adrenaline for the former, and secretin for the latter). They have more recently (26) extended these studies to the changes in R.Q., glycolysis, etc., accompanying secretion.

The Warburg-Keilin system seems to play a key role in these phenomena also. In recent studies on these same tissues I (45) have observed that the quiescent tissues have essentially cyanide- and azide-stable respiration. Upon humoral stimulation the cytochrome-cytochrome oxidase system, as in the sea urchin egg, becomes geared to the other catalysts. These cells differ from the egg in that this gearing is reversible, since the

Warburg-Keilin system progressively becomes "disconnected" again as the tissue returns to the quiescent state.

An essentially similar picture is presented by frog skeletal muscle. Stannard has shown that the increased respiratory rate attending the response to electrical or chemical (caffeine) excitation, is, in this tissue also, associated with markedly increased activity of the cytochrome-cytochrome oxidase system (68).

In connection with metabolic aspects of excitation phenomena, it is important to emphasize that the cell responds primarily to a change at its surface, produced by the electrical stimulus, by the chemical agent or, in the case of the ovum, by the spermatozoon.

The relations between cell oxidations and cell surface are evident, not only from the effects of altering the cell surface upon metabolism as above, but also conversely, from the effects of experimentally altering metabolic processes upon such properties of the cell surface as permeability and electrical potentials. These problems, of course, all touch upon the larger problems of maintenance of the normal structure of cells which depends upon oxidations and energy transfer. The problem of energy transfer in biology must be considered in conjunction with cellular organization, not only because the processes to which oxidations feed the requisite energy, such as muscular contraction, conduction, secretion, osmotic work, etc., are themselves dependent upon specialized structural organization, but also because of the very coordinated fashion in which the metabolic machinery responds to even the most abrupt changes in energy needs of the cells and to changes at the cell surface.

Studies such as those outlined above (and they can be multiplied many times) are beginning to reveal much concerning the role of cellular organization in the control and integration of cellular metabolism; they show that the incorporation of the catalytic and other components into the organized living unit imparts to them and to the ensemble, properties which cannot be predicted from even the most exhaustive studies on the isolated systems. They clearly indicate that an enzyme functioning within a living cell is characterized not only by what it is and does when isolated but also by its accessibility to substrates, by its place in the cell, and by its relation to other enzymes and to the cell's structure.

Any hypothesis which undertakes to define or describe the relation of oxidative mechanisms to cellular organization must be able to reconcile the independence of chemical reactions and their respective enzymes with their close integration, and must take into consideration such problems as have been outlined above. Thus far such

integrative mechanisms are conceivable only in morphological terms, in terms of definite intracellular structure in which the loci of various reactions bear specific and more or less fixed spatial relations to each other and to those of other cellular processes. Such a structure must be capable of maintenance in the liquid medium which protoplasm is known to be; it must be capable of mending itself after considerable disruption, as after prolonged centrifuging, osmotic swelling and shrinking, and extensive manipulation with microneedles. (The embryologists, of course, are faced with a nearly identical problem.)

One's attention is naturally first directed to surfaces, to ordered interfaces, and to the definite and often rigid structure in films of even monomolecular thickness. Even directional control may emanate from such oriented surfaces. As Hardy and Nottage (35) first showed, orientation effects may be transmitted from a surface and be capable of affecting surrounding molecules for considerable distances, and, as has been emphasized many times, long chains of oriented molecules may extend from a stable monomolecular film far into the solution.

On the basis of this principle of three-dimensional orientation under the influence of surfaces, on the basis of the occurrence of many proteins as long thread-like molecules (as demonstrated by X-ray crystallography, and studies on the chemical, physical and optical properties of protein solutions), and with supporting evidence from cytology, embryology, pharmacology and various chemical studies which demonstrate the "morphological" relationships of enzymes, a hypothesis, designated as the "cytoskeleton hypothesis" has been developed by Peters, Needham and Clark (61, 57, 16). A fuller exposition of this theory and its background may be found in the interesting book by Needham (57), particularly in the third chapter.

This hypothesis is part of a general movement in recent years towards the conception of fibre or thread molecules as the basis of protoplasmic organization (65), and hence of "that architecture which is the smallest unit in the architectonics of morphological form" (57). From the way in which it is possible to shift formed bodies and micelles about by centrifuging, with microneedles, etc., without destroying polarity, symmetry and development of egg cells, it seems that the cytoskeleton must be something easily broken yet capable of immediate repair, and that the fibers are easily detached and replaced by others, linked through such forces as residual valencies. Liquid crystals have received considerable attention in this connection, particularly those formed by proteins, since in a liquid crystal the orientation of the molecules is only temporarily affected by the

passage of a foreign object. The molecules form up again regularly behind it; the cytoskeleton is thought to behave in a similar fashion.

Briefly stated, the hypothesis, in its present state of development, is as follows. The cytoskeleton is a three-dimensional mosaic, with a protein basis throughout the cell. It consists of three parts: the surface proteins, the cytoplasmic proteins and the nuclear proteins. The surface proteins are receptor in function; the cytoplasmic proteins serve as framework and conductor; the nuclear proteins are thought to be more or less directive, and for our present purposes need not be discussed here. The enzymes, of protein nature, would naturally be closely associated with this structure, and so in large part be under the influence of activity of the mosaic.

Surface Proteins. Chemical and physical changes in the environment are thought to produce changes in cell activity *via* special receptors in the plasma membrane or by changes in the colloidal aggregation at the surface. These surface receptors comprise a very important part of the hypothesis since it is well known that many agents, drugs, etc., exert their action entirely upon the surface, having no specific action when injected into the interior of the cell, (*e.g.,* acetylcholine); many of them, such as acetylcholine, muscarine, etc., act much too quickly to be accounted for by diffusion and penetration. Quantitative experiments show that about 10,000 molecules of acetylcholine are sufficient to produce an effect on a heart cell. The quantity would cover only 1/6000 of the surface. This suggests that acetylcholine acts on special receptors. Indeed, studies on drug antagonisms, such as those of Clark and of Cook, have already begun to reveal much about the nature of these groups (16).

Cytoplasmic Proteins. This part of the cytoskeleton is thought to be capable of suffering temporary displacement without permanent upset. As previously indicated, the liquid crystal presents many analogous features. Indeed, some of the proponents of the hypothesis are convinced that the paracrystalline state seems most suited to biological functions, as it combines the fluidity and diffusibility of liquid while preserving the possibility of internal structure characteristic of crystalline solids. Liquid crystals possess internal structure lacking in liquids, and directional properties not found in gels. The mechanism by which the stimulus (applied to the surface proteins) is transmitted through the cell and to the nuclear proteins is, of course, unknown. It may actually be mechanical, using the semi-rigidity of the thin surface film; on the other hand, the speed with which the disturbances are transmitted suggests a higher sensitivity and the possibility that it is a question of electronic balance (57).

Boehm (9) has recently produced considerable direct support for such an hypothesis. He succeeded in extracting from erythrocyte ghosts a protein (stromaprotein) the molecules of which are several thousand times as long as they are thick. These fiber-molecules, according to Boehm, in addition to forming a network in the cell membrane, also form a three-dimensional framework within the cell, connected to the surface, and account for many of the physical properties of the erythrocyte.

An hypothesis as much in accordance with observed facts as this, will prove, and has already proven, useful in the study of cellular oxidations (as well as in cytology and embryology), and in the interpretation of data both from isolated systems and from intact cells. It provides a basis for understanding, in chemical terms, how independent chemical reactions can occur in various parts of the cell, yet remain at all times well coordinated. Through changes in the three-dimensional mosaic, of which the catalysts form a part, or by which they are oriented, it becomes possible to understand how a stimulus applied to the cell surface can be transmitted throughout the cell to elicit such coordinated changes in the metabolic machinery as outlined above (*e.g.,* for the sea urchin egg or salivary gland). It enables one to understand how formed bodies or micelles which have apparently no specific or fixed location in the cell can yet control so many of the reactions in the cell, and to understand how substances can reach special loci in the cell.

However, it is my wish to emphasize not so much the theory and its virtues, as the fact that it represents one of the first and few systematic attempts to apply the coordinative outlook in biochemistry. It attempts to fit the cell's individual mechanisms into the cell's organization, and to bridge the gap between chemistry and morphology. We are a long way from understanding the nature of this organization, only the upper and lower levels of which (as Needham points out) are visible to us now. But it is important to recognize that many properties, of the enzymes and systems which biochemists examine in the isolated condition (extremely important and necessary as these investigations are), become operative only in combination with this organization. The materials which biochemists today are extracting from cells must eventually be "put back", and they must be put back in their proper places and relationships.

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DISCUSSION

Dr. Clark: I think clarity will be introduced into one of the subjects you dealt with if we clearly separate the mechanisms by which the energy of one system on one side of a partition can transfer this energy to the other side from the fact that there does exist a potentiality of action due to the difference in energy of the two systems. Some of the papers of Lund to which you have referred have been confusing to me because he seems to be seeking a path of transfer of electrons. It seems to me that from the point of view of energetics this is entirely unnecessary, because if you have any mechanism anywhere in the interface by which the equivalent of an electron can get across, you may transfer the energy from the one side to

the other. If we care to speculate, that might be a reversible oxidation-reduction system in the membrane which could "stay put" in the membrane and serve as a mediator.

Dr. Korr: I quite agree. Just as a dissolved mediator can transfer energy (electrons) from one surface to another (*e.g.* colloidal platinum to cellulose) so can a mediator, fixed in a film or membrane, transfer electrons between two phases which it separates. In this way, as I pointed out, it becomes possible for oxidation-reductions to be a direct source of bioelectric potentials, measurable at non-metallic electrodes (Fig. 1). The membrane will have certain properties in common with metals, and the potentials obtained will bear direct quantitative relationships to the differences in reducing intensity or oxidation-reduction potential between the two sides. If, in addition, the membrane is also permeable to ions which will migrate in such a way as to compensate for electron migration, thus maintaining electroneutrality on each side, then a secretory mechanism is provided. Indeed, this is the mechanism postulated by Stiehler and Flexner for secretion by the choroid plexus.

There are other ways, however, in which bioelectric potentials may be directly set up by oxidation-reductions. For instance, potentials quantitatively related to ratio of oxidant to reductant (the system need not be electromotively active) will appear across the boundary between two phases in each of which the oxidant and reductant have different solubilities and mobilities. Beutner has shown that the E.M.F. between aqueous and non-aqueous phases is changed when certain organic compounds in the aqueous phase are replaced by their corresponding oxidation products. I have made measurements of potentials between aqueous solutions separated (in a U tube) by a non-aqueous phase, or separated by extremely thin lipoid-protein films. The potentials showed reproducible relations to ratios of succinate-fumarate or lactate-pyruvate. The lipoid protein film (produced according to Danielli) is of special interest because it is thought to bear many resemblances, in structure, permeability, etc., to the plasma membrane. However, the experiments are as yet unsatisfactory and difficult, because of the extremely high resistance and instability of the film.

Dr. Müller: With regard to your statement that potentials at interfaces cannot be measured, I would like to point out that there is at least one electrode suitable for this purpose, that is the dropping mercury electrode. Since I am going to speak about this electrode next week I will not describe it today, but I want to say now that we can determine the ratios of oxidant and reductant as well as the pH at the electrode solution interface, and we see from our experiments that in a well-buffered system the pH at the interface must be the same as that in the solution, while in a poorly buffered system it can be different by two or three pH units.

Dr. Barker: What is the essential difference between a surface at which activation takes place and a surface at which retardation takes place?

Dr. Korr: It is possible for both retardation and activation to take place at the same surface, depending upon the reactants. If the nature of the bonds between surface and reactant (*e.g.* at the active patches) is such that intramolecular changes are produced, by deformation of the molecule or by complex-formation, then activation of the reactant may occur. If, on the other hand, the relation of the molecular architecture of the surface to the molecular structure of the reactant is such that no activation is produced, yet permitting accumulation at the surface, then the reaction in which the adsorbed molecules participate may be retarded by the lowered molecular mobility and probability of collision. Thus many catalyst poisons are merely substances of dog-in-the-manger nature, accumulating at surfaces at which they cannot be activated, yet preventing access of molecules which can. Indeed, the surfaces of solid catalysts, including activated charcoal, metallic oxides, etc., are extremely heterogeneous, bearing active patches of great variety and specificity, in addition to the larger portions of the surface which are available for adsorption but which do not activate.

If the bonds responsible for the accumulation, say, of a reversible dye at a surface are co-valent, involving high heats of adsorption (as contrasted with molecular adsorption, comparable to condensation of liquids), then, it seems to me, the potential of the dye must be shifted, to different extents at different surfaces.

Dr. Clark: I should think if you set up certain simple cases you could measure the energy of adsorption.

Dr. Ball: With regard to the difference in potential of an oxidation-reduction system in a homogeneous and a heterogeneous medium, I should like to make these two remarks. First, you mentioned in this connection the fact that the echinochrome in the granules of the *Arbacia* egg under anaerobic conditions never appears in the reduced state. I can confirm that observation. However, I would not expect it to be reduced, because crystalline echinochrome obtained from those eggs forms a system with a very negative oxidation-reduction potential. Therefore I do not believe that this is evidence of differences due to the type of medium.

Secondly, cytochrome-c can be obtained in pure solution from heart muscle. You can also obtain a heart muscle suspension which contains cytochromes-a, -b and -c. The cytochrome-c in that suspension is presumably not in solution, because

I have never been able to wash all of the cyto-chrome-c out of such a muscle suspension. The oxidation-reduction potentials of cytochrome-c in this suspension or in pure solution, however, agree within 5 to 10 millivolts.

Dr. Barron: I want to add to the statement regarding the possibility of two enzymatic sluggish systems reacting without a mediator. Borsook showed that the lactate-pyruvate and succinate-fumurate systems exchanged electrons when an electroactive mediator was introduced. It seems that the assumption is made that in living cells the electron exchange might occur without medi-ators, because none were found. It is now well known that such electroactive mediators do exist. We have, for example, the alloxazins which are presumably electron mediators; so we do not need to go to the hypothesis that sluggish systems react with one another without the mediation of elec-troactive systems.

Dr. Baumberger: Have you had any exper-ience with stirring the adsorbent on which some oxidation-reduction system is adsorbed and thus bringing it into contact with the electrode at very high velocity and seeing whether or not you then have a modified potential? The idea I have in mind is that possibly the adsorbed oxidation-re-duction system would impress upon the electrode a potential which would differ from the potential impressed on it by the unadsorbed oxidation-re-duction system, and therefore a modified potential would be obtained by the stirring. There are ex-periments in which sluggish systems come more rapidly into equilibrium with the electrode by stirring.

Dr. Korr: Yes. In my experiments in which the potentials are markedly shifted by the addi-tion of adsorbent, once the potential has reached its new level, it remains perfectly constant, ir-respective of the rate of stirring by nitrogen gas; there is no further change in potential even if the adsorbent be permitted to settle, away from the electrode, by stopping the bubbling of the nitrogen. This is true even of very dense suspensions.

That adsorbed systems will not exchange elec-trons with electrodes with which they briefly come in contact is not surprising; we need only recall that even finely divided platinum (platinized asbestos) fails to reduce adsorbed dye in the presence of hydrogen, despite the great increase in surface and much greater frequency of collision between platinum and adsorbed dye; even if the finely divided platinum and the dye are fixed in very close proximity, perhaps in contact, on the same surface, as in Kautsky and Baumeister's ex-periments, there is apparently still no reaction.

Dr. Baumberger: Of course in that case isn't it conceivable that the methylene blue adsorption is very much greater on the adsorbent than on platinum, and therefore the methylene blue may not adsorb on the platinum? There must be an adsorption of methylene blue and of hydrogen on the platinum at the same time in order to have the transfer and reduction occur, whereas in some other adsorptions the relative variance may not be so great.

Dr. Korr: That is precisely the explanation—the reactants must be present on the surface of the catalyst together. In the experiments of Kautsky and Baumeister the rate was entirely dependent on how much methylene blue was reaching the platinum-hydrogen surface from solution.

THE RELATION OF OXIDATION-REDUCTION POTENTIALS TO CHEMICAL STRUCTURE

Paul W. Preisler

Attempting to correlate the properties of organic compounds with their structure has always been a fascinating occupation, chiefly because the numerous exceptions immediately encountered offer a new challenge to the searcher for the basic laws to form more inclusive hypotheses or more general rules.

The present review of the relation of oxidation-reduction potentials to chemical structure is necessarily limited to readily reversible systems of known structure whose potentials can be directly measured in buffered solutions by electrodes or estimated by their reactions with other reversible systems of known oxidation-reduction potentials. The material outlines only the general rules which have been developed and does not aim to include every compound or type already studied, because some systems are only isolated cases representing a particular unrelated structure and because much of the data available in the literature cannot be logically used for comparison owing to the great diversity of experimental conditions under which they were obtained. Many attempts to correlate such material by calculations and corrections involved uncertainties obviously greater than the effects actually produced.

The possibilities of studying organic structural relations through the measurement of oxidation-reduction potentials were early recognized, and many type systems and substitution compounds were prepared and measured. Thus by a quite thorough study of the quinone-hydroquinone systems of various types and substitutions, Conant and Fieser (13, 20) and their collaborators have obtained data valuable in elucidating the nature of the ring structures of organic compounds such as benzene, naphthalene, phenanthrene, anthracene, and others. Their studies have also given indication of the predominating tautomer of a tautomeric equilibrium in the cases of some hydroxy-quinone (18) and amino-quinone (21) systems, and have revealed some of the effects of substitutions upon the fundamental quinone-hydroquinone type (13). Clark, Cohen, and their collaborators, in their search for suitable indicators, have also contributed much valuable information to the general problem of structure and potentials through their results with the indophenols (5, 9, 10, 11, 24, 25), the indigo sulfonates (38), and the many other type structures (1, 2, 6, 7, 8, 12, 30, 36, 37).

With the exception of these groups of investigators, systematic, though limited, investigations for the purpose of studying the fundamental organic structural relationships have been made only in isolated instances, as for example, by LaMer and Baker (26) on the quinones, by Billmann and Lund (3) on the alloxans, and by Preisler and Hemplemann (32, 33) on the thioindigos and the *para*-isomer of pyocyanine, N-methyl-β-oxyphenazine. It is to be regretted that many observers, limiting their observations to conditions dictated by their interest in highly specialized problems, made only a few measurements on their material, whereas with but little additional expenditure of time they might have extended their measurements and contributed much to this important and fundamental phase of the subject of oxidation-reduction potentials.

The development of the semiquinone concept by Michaelis and his collaborators (27, 28) and by Elema (16, 17), growing out of their work on the pigment pyocyanine and extended to other systems by them and other investigators, has opened another field for exploration which promises to give further insight into the problem of the fundamental properties of organic structural relationships.

At the beginning of the experimentation in the field of oxidation-reduction potentials, essentially all systems were considered as having a two-equivalent change between oxidant and reductant with the change occurring in a single two-valent step. The discovery of the semiquinones showed that in some cases the two-equivalent change occurred in essentially two one-valent steps. The extension of investigations (31) of compounds having more than a two-equivalent change offers some interesting possibilities. Preisler and Hill and collaborators (31, 34) have measured some of the stages of the reversible reduction of triquinoyl to hexahydroxy-benzene, a system involving ultimately six equivalents on the same molecule, and the various phenazine type condensation products of this series and its related series, leuconic acid to hydrocroconic acid (31). Such investigations should add much information also valuable to the formulation of the principles of catalysis of oxidation and reduction reactions by reversible oxidation-reduction systems as discussed by Shaffer (35).

From the above brief historical review with indications as to the present trends of investigations and from the attempted classification of systems which is to follow, it will be realized that we are still far from formulating any very inclusive laws or rules regarding structure and potentials.

The great number of applications of the oxidation-reduction potential methods and techniques to biological problems involving oxidations and

reductions is ample proof of their increasing importance to biology. In the final analysis, all such reactions are chemical in nature, and if these chemical reactions were thoroughly understood many of the perplexing biological problems would become clear. The study of simple chemical structures is one phase of the attack upon biological problems.

All reversible organic oxidation-reductions may, for convenience in explaining, be considered essentially additions of hydrogen (or electrons, depending on the ionic condition) to the atoms adjacent to a double bond or to the terminal atoms of a conjugated series of single and double bonds. Thus in adding HH to nitrosobenzene, $C_6H_5 . N:O$, to give phenylhydroxylamine, $C_6H_5 . NH . OH$, the double bond between $-N:O$ has been converted into a single bond $-N . O$ HH. In the same manner azobenzene $C_6H_5 . N:N . C_6H_5$ can be reduced to hydrazobenzene, $C_6H_5 . NH . NH . C_6H_5$, by addition to the double bonded nitrogens $-N:N-$ giving $-N . N-$ HH. These are examples of 1 - 2 addition of hydrogen. The addition of hydrogen to two carbon atoms connected by a double bond differs from these in being essentially irreversible in the thermodynamic sense. No oxidation-reduction potentials have ever been measured for HH addition to $-C:C-$ and the reduction usually requires hydrogen to be present in the dissolved state in the presence of a suitable metallic catalyst.

Certain compounds of aromatic character which have conjugated double bond systems are reversibly oxidized and reduced. *Ortho*-benzoquinone, $(C_6H_4)(O_2)$, has the conjugated series

$$-\,C-C-\quad\text{which adds HH}\quad -\,C:C-$$
$$||||\qquad\qquad\text{to give}\qquad\qquad||$$
$$OO\qquad\qquad\qquad\qquad\qquadOO$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad||$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquadHH$$
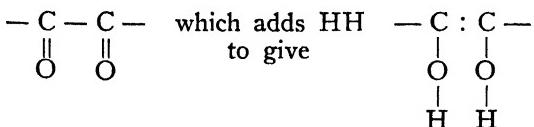
with a rearrangement of the bonds of the conjugated series. Since the HH is added at the ends of a series of 4 atoms it is termed a 1 - 4 addition. The addition of HH to an aliphatic diketone is apparently not reversible in a thermodynamic sense. *Para*-benzoquinone adds the HH in a 1 - 6 manner,

$$-\,C-C:C-C-\qquad -\,C:C-C:C-$$
$$||||\qquad||$$
$$OO\;\;\text{giving}\;\;OO$$
$$\qquad\qquad\qquad\qquad\qquad||$$
$$\qquad\qquad\qquad\qquad\qquadHH$$
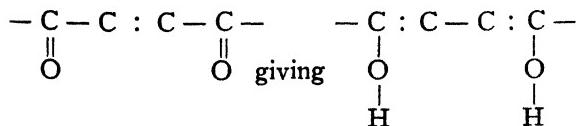
Reversible oxidation-reductions of organic compounds may be grouped into classes depending on the nature of the addition of the HH of the reduction, and these classes further subdivided on the basis of the nature of the structure of the remainder of the molecule. When this is done, it is noticed that of the systems recorded in the literature, the greatest number by far are the 1 - 6 additions which are represented by many type structures, the 1 - 4 additions are limited almost entirely to *ortho*-quinones, and the systems with other spacial additions are few in number.

The following tabulation of systems is according to type of addition without attempting to indicate the terminal atoms of the active grouping or substitutions in the remainder of the molecule adjacent to the active grouping. This list is not complete but serves to illustrate the types encountered.

1 - 2 additions

nitroso-benzene to phenyl-hydroxylamine
azobenzene to hydrazobenzene
alloxan to dialuric acid

1 - 4 additions

ortho-benzoquinone to 1, 2 dihydroxy-benzene
1, 2 naphthaquinone to 1, 2 dihydroxy-naphthalene
1, 2 phenanthrenequinone to 1, 2 dihdroxy-phenanthrene
9, 10 phenanthrenequinone to 9, 10 dihydroxy-phenanthrene
1, 2 anthraquinone to 1, 2 dihydroxy-anthracene
pyocyanine to N-methyl-α-hydroxy-phenazine

1 - 6 additions

para-benzoquinone to hydroquinone
1, 4 naphthaquinone to 1, 4 dihydroxy-naphthalene
1, 4 anthraquinone to 1, 4 dihydroxy-anthraquinone
9, 10 anthraquinone to 9, 10 dihydroxyanthraquinone
quinonimine to aminophenol

The following named oxidized states to their corresponding leuco derivatives.

indophenols	oxazines
indamines	thiazines
indigos	eurhodines
thioindigos	safranines
alloxazines	*iso*-alloxazines

Examples of higher additions are the reduction of *amphi*naphthaquinone to 2, 6 dihydroxynapthalene, a 1 - 8 addition; viologen to γ, γ' dipyridyl, and diphenoquinone to p, p' dihydroxydiphenyl, 1 - 10 additions.

All compounds related to the reversible oxidation-reduction types described are not necessarily measurable at electrodes by the usual technique, but some can be estimated by their effects upon reversible indicator systems of known potential. For purposes of classification of reversible oxidation-reduction systems there are available several constants:

E_o for the system representing the potential of the equimolecular mixture of oxidant and reductant at pH 0.

pK for the oxidant, the reductant and the semiquinone, the semiquinone formation constant and

the dimer formation constants. These values may be expected to vary with the concentration of the components of the system, the temperature, the concentration of the buffer or its ionic strength, the total concentration of the components, the pH of the buffer, and other factors of lesser importance.

E_0 is the constant most frequently used for comparison, generally without regard for the ionic condition of the components at this pH. A complete classification of the E_0 of the various predominating ionic species on the basis of the values of E'_0 extrapolated to pH 0 is being made and will be published later.

The ionizations of the components, best represented by pK values, which are responsible for the changes in slope of the E'_0 — pH curves and which are apparently a factor in influencing the semiquinone formation (32), have not been used much for comparative purposes.

The number of systems for which the semiquinone formation and the dimer formation has been determined is still too small for significant classification purposes.

The simplest organic structures which can be reversibly reduced by the addition of HH are
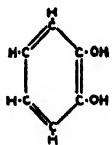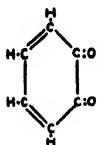
$E'_0 = +0.784$

ortho-benzoquinone to 1, 2 dihydroxybenzene
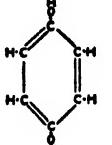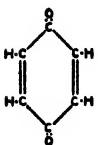
$E'_0 = +0.713$

para-benzoquinone to 1, 4 dihydroxybenzene

Ortho- structures (1 - 4 additions) are generally more positive than the corresponding *para*- structures (1 - 6 additions).

Systems having these active groups joined to a ring structure have oxidation-reduction potentials which are less positive than the simple system
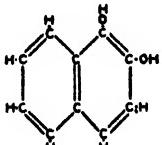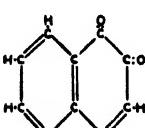
$E'_0 = +0.577$

1, 2 naphthaquinone to 1, 2 dihydroxynapthalene
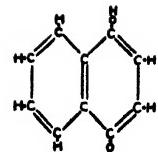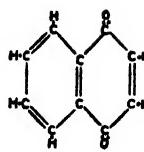
$E'_0 = +0.483$

1, 4 naphthaquinone to 1, 4 dihydroxynapthalene

The effect is more marked when two rings adjoin the active group:

$E'_0 = +0.456$
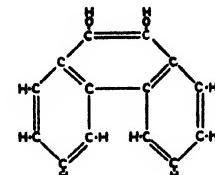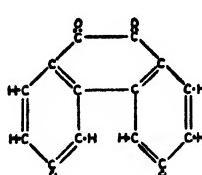
9, 10 phenanthrenequinone to 9, 10 dihydroxyphenanthrene
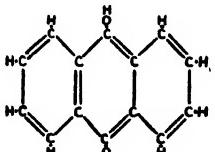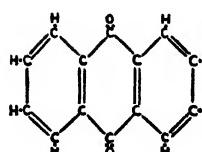
$E'_0 = +0.140$

9, 10 anthraquinone to 9, 10 dihydroxyanthracene

The E'_0 values cited or calculated are from the work of Conant and Fieser (14, 15) and Fieser (18) and were obtained in various mixtures of H_2O, C_2H_5OH, HCl, and LiCl, and, although not strictly comparable, are illustrative of the gross effects to be expected. The rings may have other spatial relationships to the active groupings as, for example, in the *ortho*-quinone types, 1, 2 or 3, 4 phenanthrene-quinones, or 1, 2 or 3, 4 anthraquinones, and in these cases the potentials are also lower but with certain irregularities as noted by Fieser (19). Similar results are recorded with active groupings to which heterocyclic rings are attached.

The effect of substitution for the H atoms attached to the ring structures may be either to raise the oxidation-reduction potential above that of the simple system or to lower it. The following differences recorded in the table were obtained by subtracting the E'_0 of the simple system from the E'_0 of the substituted system measured under similar conditions (Table I) (2, 13, 14, 15, 22).

Summarizing the data, the following conclusions may be drawn: —CH_3, —OH, —NH_2, —OCH_3, —$NH(CH_3)$, —$N(CH_3)_2$, —$NH(C_6H_5)$ and similar groups lower the po-

TABLE I

Effect of substitution on various quinones.

(The figures represent the voltage of the substituted derivative above (+) or below (−) the unsubstituted type structure at or near pH 0.)

Substituent and position		o-quinone (1, 2)	p-quinone (1, 4)	o-naphthoquinone (1, 2)	p-naphthoquinone (1, 4)
(CH$_3$)	2		−0.055		−0.076
(CH$_3$)	4			−0.045	
(CH$_3$)	6			−0.023	
(CH$_3$)$_2$	23				−0.144
(CH$_3$)$_2$	25		−0.110		
(CH$_3$)$_2$	26				−0.079
(CH$_3$)$_2$	27				−0.077
(CH$_3$)$_2$	37			−0.041	
(CH$_3$)$_3$	235		−0.173		
(CH$_3$)$_4$	2356		−0.247		
(NH$_2$)	2				−0.210
(NH$_2$)	4			−0.251	
NH(CH$_3$)	2				−0.252
NH(CH$_3$)	4			−0.283	
NH(C$_2$H$_5$)	4			−0.279	
NH(C$_6$H$_5$)	2				−0.198
N(CH$_3$)$_2$	2				−0.181
(OH)	2		−0.112		−0.127
(OH)	8				−0.030
(OH)$_2$	25		−0.256		
(OH)$_2$	26				−0.180
(OH)$_2$	58				−0.122
(OH)$_3$	258				−0.240
(OCH$_3$)	2				−0.131
(OCH$_3$)	4			−0.143	
(OCH$_3$)$_2$	25		−0.234		
(OCH$_3$)$_2$	26		−0.186		
(Cl)$_{1-4}$		+0.038	+0.018		
(SO$_3$Na)	2				+0.069
(SO$_3$Na)	4			+0.060	
(COOH)	2		+0.097		
(COOH)	4	+0.091			
(COOC$_2$H$_5$)	2		+0.098		
(COOC$_2$H$_5$)	4	+0.091			

tential of the simple type system, and —SO₃H, —COOH, —COOC₂H₅, and halogens raise the potential. Substitution for an H connected to the C of a ring adjacent to the active group has less effect than if the C were in the same ring. Each additional substitution for another H generally causes a nearly proportional change.

Systems having more than two oxidation equivalents per mole have not been extensively studied. Fieser and Peters (23) reported a potentiometric oxidative titration of dinaphthyl-dihydroquinone
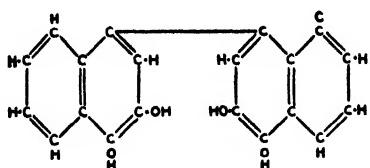
in acid alcoholic solution which gave two approximately two-valent curves, and Cannan (4) showed that the plant pigment, hermidin, of undetermined structure, also gave two essentially two-valent curves.

Preisler and Hill and collaborators (34) reported the potentials of the reduction of rhodizonic acid through tetrahydroxyquinone to hexahydroxybenzene which requires two equivalents for each step. The complete series including the oxidation of rhodizonic acid to triquinoyl presents the interesting case of six equivalents on the same ring.
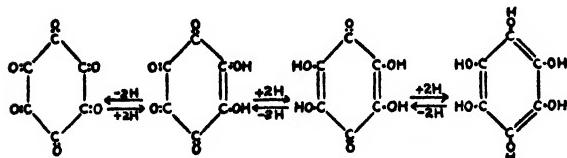
The unusual reactivity of the compounds and low solubilities make difficult the measurement of the step to triquinoyl. In the region of pH from 5 to 6, the two essentially two-valent curves determined at higher or lower pH of the steps, rhodizonic acid → tetrahydroxyquinone → hexahydroxybenzene, merge into one single S-shaped curve. The region of this curve is so short that it could not be established whether the curve would ever reach that form required by theory for a single four-valent step.

The possibility that this system reacts through a single four-valent step raises the interesting point of this system acting as a catalyst for the oxidations by molecular oxygen by taking up the four equivalents of molecular oxygen (O₂) at once and releasing them singly or in pairs through the two systems or their semiquinones. Possibly the ap-

plication of the polarographic technique with the dropping mercury electrode as described by Müller and Baumberger (29) might give some insight on the nature of this valence change.

With a compound such as triquinoyl, which on reduction may yield isomeric derivatives, the complete structural configurations cannot be definitely established, since it may be considered as two *ortho*-quinones and one *para*-quinone or three *ortho*-quinones; the studies in progress (31) on the phenazine type condensation products may lead to a solution of this complex problem.

For the biologist possibly the chief value in the study of the relation of oxidation-reduction to chemical structure is in getting a better insight into the nature of the materials and reagents used in the various oxidation and reduction experiments and their probable effect on other reactants. Structure has a large influence on toxicity, diffusion rates, ionic state at a particular pH, solubility, color, and many other factors. Many times a slight change in the structure of a substance produces a desired result. Much time might be saved by a proper selection, or by the preparation of more suitable materials.

Tables of indicators are prepared in most biological tabulations by calculating the potential at pH 7, listing them in the order of decreasing potentials, usually without a single reference to the chemical structure or even a precaution as to the use of the dyes in question. Oxidation-reduction systems when used as indicators must be considered as reactants, and an estimation of potential by dyes should include several measurements employing separately at least two dyes of different structure, at two different concentrations of each.

A few additional instances in which a knowledge of the chemical structure may be useful will be presented. In general, two classes of substances will be encountered, one essentially reversible in the thermodynamic sense and the other essentially irreversible.

If the system present is reversible, the potential may be useful (*1*) for indicating the general character of the substance, thereby allowing for more intelligent selection of methods used in its isolation; (*2*) in determining the amount present by employment of the most advantageous reagent, that is, one which will not affect other substances (for example, the estimation of vitamin C); and (*3*) in serving for its identification without isolation. When a substance has been isolated, information on the predominating tautomer may be obtained or the position of a substituent group may be established. New constants for classification or identification may also be made available.

If the system to be investigated is irreversible in character, the knowledge may aid in the selec-

tion of suitable oxidizing or reducing agents or in the control of reaction rates under certain conditions and possibly for identification if such factors have been carefully determined.

The entire subject of the relation of chemical structure and oxidation-reduction potentials is apparently still in its formative stages, but even with the limited data available some general rules have been formulated, the establishment of the validity of which will require considerable additional experimentation.

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DISCUSSION

Dr. Hellerman: Preisler's comment concerning oxidation-reduction "indicators" is worth reemphasis. The late Dr. Abel in speaking of epinephrine and similar substances, said that such materials must be "used with intelligence". I think the same thing may be said of the so-called oxidation-reduction indicators; they must be used with intelligence. In elementary physical chemistry we used to be taught that one difference between a dye and an "indicator" was in rapidity of action: if the dye changed color rapidly with a change in hydrogen ion concentration it was an indicator. We often indiscriminately call certain dyes oxidation-reduction indicators when under some conditions they are truly indicators, and under other conditions they are not such at all. Sometimes, instead of being reduced or oxidized, they undergo other changes. In the complexities of biological work, especially, must their behavior be interpreted with care.

THE ROLE OF FLAVOPROTEINS IN BIOLOGICAL OXIDATIONS

Eric G. Ball

The recognition in recent years of the close relationship existing between vitamins and oxidative enzymes may well be said to have begun with the discovery by Warburg and Christian (44) in 1932 of the first flavoprotein. The interesting story of the identification of the prosthetic group of this oxidative enzyme as riboflavin phosphate and the recognition of its vitamin activity has been well told in previous reviews (47-53). Within the past year our knowledge of the role of the flavoproteins in biological oxidations has been enormously widened. No less than five roles are now assigned to flavoproteins in contrast to but one known heretofore. A discussion of these roles and the flavoproteins concerned will be presented in this paper.

Prosthetic Groups

Flavoproteins are now known which possess one of two prosthetic groups. As mentioned above, the first to be discovered was riboflavin phosphate. It is a mononucleotide with the structure represented by (1) in Fig. 1. The other, which appears to occur more abundantly, is a dinucleotide and was first isolated and identified by Warburg and Christian (45) in 1938. According to these workers it is composed of one adenine, one flavin, two pentose, and two phosphoric acid molecules, and may be looked upon as formed by the combination of riboflavin phosphate and adenylic acid with the elimination of one molecule of water (see Fig. 1). Abraham (1) has recently claimed that
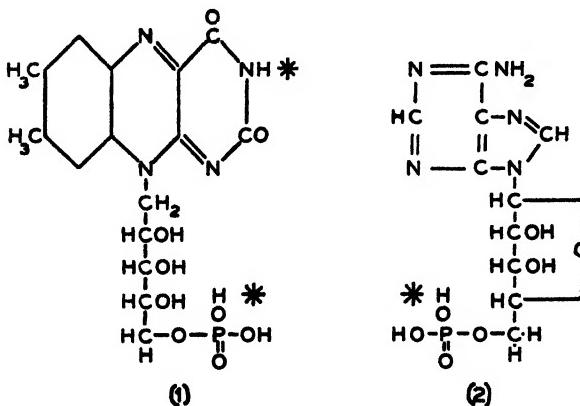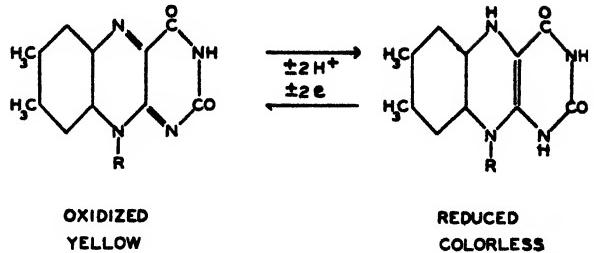
Fig. 2. The prosthetic group as an oxidation-reduction system.

the dinucleotide as prepared by the method of Warburg and Christian yields on acid hydrolysis adenosine-5-phosphoric acid and riboflavin phosphate. His evidence is based on suitably controlled enzymatic tests in which these mononucleotides are known to be active as coenzymes. The exact nature of the linkage joining these two mononucleotides is not known, though presumably it occurs through one or both of the phosphoric acid groups. Only one flavoprotein (44) is known which contains riboflavin phosphate as its prosthetic group. Its discoverers, Warburg and Christian (46), have now raised the question as to whether it may not be an unnatural product resulting from the loss of adenylic acid from the prosthetic group during the course of the isolation. This would mean, then, that the dinucleotide is the only true prosthetic group of flavoproteins.

The resemblance in structure of flavin adenine dinucleotide to the so-called diphosphopyridine nucleotide is indeed striking. The two compounds differ only in the nature of their functional groups; in one it is an isoalloxazine ring, in the other nicotinic acid amide. One wonders whether the flavin analogue of the triphosphopyridine nucleotide will also be found in nature. The pyridine and flavin dinucleotides, despite their structural similarities, appear, however, to differ markedly with regard to their affinities for their respective proteins (cf. Negelein and Brömel, 35). The flavin dinucleotides appear to enter into a much tighter union with their protein carriers. The exact nature of the linkage between the flavin prosthetic group and the protein molecule is not known. There is evidence, however, that at least two types of groups in the flavin mononucleotide are concerned in the linkage. One of these appears to be the phosphoric acid residue, since riboflavin alone cannot effectively replace riboflavin phosphate as a coenzyme in enzymatic tests (Theorell, 52; Kuhn and Rudy, 29). The other grouping appears to lie in the isoalloxazine ring and Kuhn and Boulanger (28) have suggested that

(1) = FLAVIN NUCLEOTIDE

(1)+(2) = FLAVIN-ADENINE DINUCLEOTIDE + H$_2$O

✱ = POTENTIAL PROTEIN LINKING GROUPS

Fig. 1. The chemical structure of the flavin prosthetic groups.

the NH group, which is starred in Fig. 1, is concerned in the linkage. They muster the following evidence in support of this proposal.

1. The oxidation-reduction potential of the flavoprotein is some 120 mv. more positive than that of the prosthetic group alone. Since changes in the side chain of the prosthetic group, such as removal of the phosphoric acid group, do not affect its potential but substitutions in the isoalloxazine ring do, one conclusion which may be drawn is that the protein is attached to the ring.

2. Whereas the flavoprotein does not fluoresce, the free prosthetic group does. In alkaline solution, however, the fluorescence of the prosthetic group is quenched and here also the NH group undergoes a pH change. The failure of the flavoprotein to fluoresce, therefore, suggests that here also the NH group of the prosthetic group is altered.

3. If the NH group concerned is methylated, riboflavin loses its vitamin activity. This may be interpreted as signifying that the vitamin activity is correlated with its ability to link with protein.

Although all of the above evidence was presented for the flavin mononucleotide and its corresponding flavoprotein, there seems to be no reason why it should not hold also for the newly discovered flavoproteins which contain a dinucleotide as a prosthetic group. In their case, however, there exist two phosphoric acid residues, both of which may be concerned in the linkage. Ball (8) has determined the oxidation-reduction potential of a flavin adenine dinucleotide sample kindly furnished by Prof. Warburg and finds at pH 7.8 and 30° C. $E'_0 = -0.250$ v. This is only 18 mv. more negative than the value given by Michaelis, Schubert, and Smythe (33) for riboflavin and about equal to that given by them for lumiflavin under the same conditions. An attempt to determine (8) the oxidation-reduction potential of a xanthine oxidase preparation (6) which appears to contain a flavoprotein with a dinucleotide prosthetic group yielded values which indicated the presence of a system with an E'_0 lying at about —0.08 to —0.09 volts at pH 7.8 and 30° C. This value lies within the potential region given by Kuhn and Boulanger (28) for the flavoprotein studied by them. The prosthetic group obtained from the xanthine oxidase preparation gave a value for $E'_0 = -0.230$ v. under the same conditions. Though these values are the results of preliminary experiments they serve to indicate that the same positive potential shift may also hold for the dinucleotide compounds when conjugated with a protein.

Another indication that the protein and prosthetic group may be linked through the isoalloxazine ring is the fact that even in the visible range
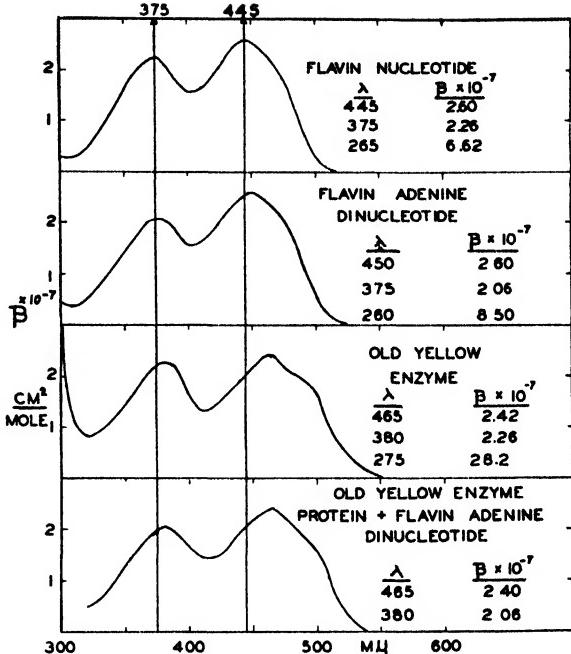
Fig. 3. The absorption spectra of the flavin prosthetic groups and two yeast flavoproteins. The data for the flavin prosthetic groups is taken from Warburg and Christian (45). It is assumed that the absorption spectrum of the flavin nucleotide is identical with that of riboflavin given by these workers. The data for the old yellow enzyme are those given by Haas (25) at pH 7.4. Theorell (52) gives similar values except that in the region 270 mμ he reports $\beta = 11.1$. The value given here for 275 mμ was obtained from Haas in a personal communication. The data for the synthetic flavoprotein (see text) are for pH 7.4 as given by Warburg and Christian (46). All values were apparently determined at 100m temperatures.

$$\beta = 1/c.1/d.\ln \frac{i_o}{i} \left[\frac{cm.^2}{Mole} \right].$$

the absorption spectra of some of the flavoproteins differ considerably from those of their prosthetic groups. Such a difference can be seen in Fig. 3 where the absorption spectra of the flavin mononucleotide and its corresponding flavoprotein (labeled "old yellow enzyme") are given. The protein compound shows a peak in the visible that lies 20 mμ farther toward the red and is slightly lower than that for the prosthetic group alone. The band centered at 375 mμ shows less change. It is of interest that if the protein part of the "old yellow enzyme" is combined with the flavin dinucleotide, as can be done in a manner to be described below, a similar shift in the absorption spectra occurs (see Fig. 3). The fact that the absorption spectra of the flavin mononucleotide and the flavin adenine dinucleotide do not differ as greatly in the region 300-500 mμ indicates that
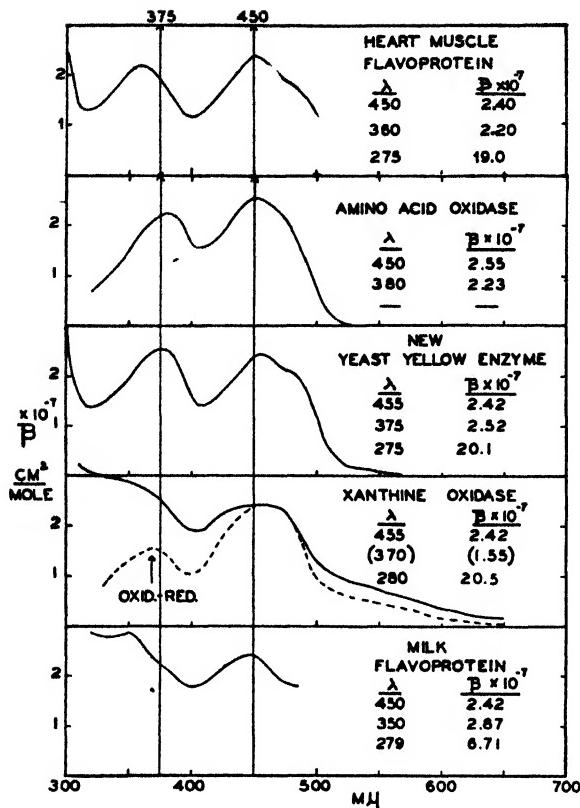
Fig. 4. The absorption spectra of various flavoproteins. Data for the heart muscle flavoprotein as given by Straub (40) and for the milk flavoprotein at pH 7.0 as given by Corran and Green (11) were recalculated in terms of β by assuming β for 450 mμ equal to 2.40 × 10⁷. The data for the amino acid oxidase are taken from Negelein and Brömel (35); for the new yeast yellow enzyme at pH 7.4 from Haas (25); and for xanthine oxidase at pH 7.4 from Ball (6). The lines drawn at 375 and 450 mμ correspond to the absorption spectrum peaks for the free flavin adenine dinucleotide. All values were apparently determined at room temperature.

the changes cannot be attributed to linkage through the side chain alone.

The flavoproteins whose absorption spectra are shown in Fig. 4 are all believed to possess flavin adenine dinucleotide as their prosthetic groups. Of these compounds, a xanthine oxidase preparation, which is possibly identical with milk flavoprotein (see below) shows the most pronounced difference between its absorption spectrum and that of its prosthetic group. The absorption spectrum of the flavin prosthetic group of this preparation is practically identical with that for flavin adenine dinucleotide. The protein compound, however, differs from it most pronouncedly, especially by its absorption in the region 500-600 mμ. This flavoprotein also differs most markedly in its absorption spectrum from that of the other known

flavoproteins. Solutions of this xanthine oxidase preparation have a golden brown color in contrast to the pure yellow color of other flavoprotein solutions. This would seem to indicate that perhaps a different type of linkage exists between the prosthetic group and protein in this flavoprotein.

Additional evidence that the protein and prosthetic group are linked through the isoalloxazine ring is furnished by the case of the amino acid oxidase. In this flavoprotein the reduced prosthetic group is more tightly bound to the protein than the oxidized form (see below). Such a difference of affinity for the protein by the oxidized and reduced form of the prosthetic group speaks for the type of linkage under consideration since oxidation-reduction changes in the prosthetic group are centered in the isoalloxazine ring.

As stated above, the linkage between the flavin nucleotides and their protein carriers is a fairly stable one. The prosthetic groups may be split off from solutions of the flavoproteins, however, by the application of heat, or the addition of acid or alcohol. The protein part is, however, denatured during these processes. In some cases, however, a separation of the two components without loss of protein activity may be obtained by dialysis of a weakly acid solution (cf. Theorell, 42, 52), or by a simple method first introduced by Warburg and Christian (45) whereby acidification is carried on in the presence of sufficient ammonium sulfate to precipitate the protein as acidification proceeds. When separation is effected by the latter means neither fraction is active alone but the mere mixing of both fractions in a neutral medium regenerates the flavoprotein and full activity is regained. By these means Warburg and Christian (46) have been able to combine either the flavin mononucleotide or the dinucleotide with the same protein fraction.

The role of the flavoproteins in biological oxidation appears to be that of mediators between two sets of compounds whose direct reaction with one another appears to be sluggish. The flavoproteins are reduced by one set of compounds and oxidized by the other. The substances by which they are reduced are far better known than the substances by which they are oxidized. In such a role it is the isoalloxazine nucleus that may be termed the functional group. A reversible oxidation-reduction system therefore exists which may be represented as in Fig. 2. The colored flavoproteins in the presence of their substrates thus may undergo a reduction to a colorless compound and become reoxidized either by oxygen or other hydrogen acceptors. In some cases such a role for the flavoproteins has been questioned and this will be dealt with when the individual flavoproteins are discussed below.

Reduced Pyridine Nucleotide Dehydrogenases

Without doubt the most important known role of flavoproteins in biological oxidations is their participation as mediators in oxidations initiated by means of the pyridine nucleotides. Two pyridine nucleotides are known and will be referred to here as diphospho- and triphosphopyridine nucleotides. Their mode of action, which has been fully discussed in an earlier review (7), may best be explained by using as an example the system which led Warburg and Christian (44) to the discovery of the triphosphopyridine nucleotide [$Py(PO_4)_3$].

This new yellow enzyme apparently possesses flavin adenine dinucleotide as its prosthetic group. It reacts more rapidly with the reduced triphosphopyridine nucleotide than does the old yellow enzyme. On the other hand the rate of reaction of the reduced form with molecular oxygen is far slower even than that of the old yellow enzyme. The reduced form, however, reacts rapidly with methylene blue and this fact was made use of to couple it with molecular oxygen in the isolation test. The substance or substances in the living cell which take the place of methylene blue are not known. Cytochrome-c does not appear to

$$\text{Hexose monophosphate} + Py(PO_4)_3 \xrightarrow{\text{protein}} H_2Py(PO_4)_3 + \text{phosphohexonic acid}$$

$$H_2 \text{ flavoprotein} + Py(PO_4)_3 \longleftarrow H_2Py(PO_4)_3 + \text{flavoprotein}$$

$$H_2 \text{ flavoprotein} + O_2 \longrightarrow H_2O_2 + \text{flavoprotein}$$
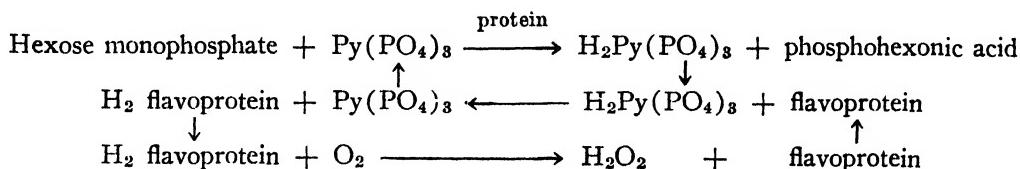
The substrate undergoing oxidation is hexose monophosphate. Though not oxidized by molecular oxygen, it is oxidized by the pyridine nucleotide in the presence of a specific protein to phosphohexonic acid and the pyridine nucleotide in turn is reduced [symbolized here as $H_2Py(PO_4)_3$]. The reduced pyridine nucleotide also can not be oxidized by molecular oxygen but can be reoxidized by a specific flavoprotein. The reduced flavoprotein formed can, however, be oxidized by oxygen so that the overall result is the oxidation of the sugar aldehyde group by oxygen to the corresponding sugar acid. Both the pyridine nucleotide and the flavoprotein thus react as cyclic catalysts and participate over and over again in the reaction. The flavoprotein that functions in this system is obtained from yeast and was the first of these protein compounds to be isolated, as mentioned earlier. Its prosthetic group is flavin mononucleotide and it will be referred to here as the old yellow enzyme. In the isolated system where an oxygen atmosphere is employed the reduced flavoprotein is readily oxidized. Theorell (43) has, however, pointed out that its rate of oxidation at oxygen tensions existing in living tissue is so slow as to make it doubtful that this is the normal pathway of its reoxidation. He finds that reoxidation occurs more rapidly by means of cytochrome-c than by oxygen at such low partial pressures and suggests that the cytochrome system may be concerned in the reoxidation of this reduced flavoprotein in vivo.

Warburg and Christian (46) have, however, questioned the existence in vivo of this flavoprotein since Haas (25) in the same laboratory has isolated another flavoprotein from yeast which may be substituted for it in the above system.

react directly with the reduced form of this new yellow enzyme.

The question now naturally arises as to whether these two yeast flavoproteins may not possess the same protein moiety and differ only in their prosthetic groups. Haas (25) presents evidence to indicate that the two proteins are also different. Though unable to dialyze away the prosthetic group of the new yellow enzyme, he was able to separate the two components without their destruction by acidification of an impure enzyme preparation in the presence of ammonium sulfate. Addition to the protein so obtained of either the separated prosthetic group or flavin adenine dinucleotide restored equally well the activity in the test system. The addition of flavin mononucleotide which should produce the old yellow enzyme if the protein parts are identical was, however, without effect. This is in marked contrast to the behavior of the protein part of the old yellow enzyme which, as shown by Warburg and Christian (46), can combine with either the flavin mononucleotide or dinucleotide to give an active flavoprotein. Further evidence that the proteins are different, as pointed out by Haas (25), is that the new yellow enzyme shows an absorption peak centered at 455 mμ (see Fig. 4) whereas the synthetic flavoprotein, composed of flavin adenine dinucleotide and the protein part of the old yellow enzyme, possesses an absorption peak centered at 465 mμ (see Fig. 3). Though the protein parts of the two yellow enzymes are not identical they possess nearly the same molecular weights. From its flavin content Haas calculates the molecular weight of the new yellow enzyme to lie between 60,000 and 65,000. The old yellow enzyme, according to Theorell (52), has a molecular weight between 75,000 and 80,000.

If the old yellow enzyme with its flavin mono-nucleotide group is, as Warburg and Christian (46) suggest, an unnatural product resulting from the splitting off of an adenylic acid group during its isolation, then, since its protein part is not identical with the new yellow enzyme, there must exist in yeast two different yellow enzymes both with flavin adenine dinucleotide as their prosthetic groups.

Both of these flavoproteins play a role in a system of which triphosphopyridine nucleotide is a part. Whether they may play similar roles in other systems where the diphosphopyridine nucleotide acts as the coenzyme, is difficult to state at the present time. Some confusion has resulted in this field due to the description of tissue preparations, called "diaphorase" by von Euler et al. (16, 17) and "coenzyme factor" by Dewan and Green (14), which brought about the oxidation of reduced diphosphopyridine nucleotide but which both these groups of workers claimed was not due to flavoprotein. Straub (40) has recently, however, isolated a flavoprotein from heart muscle which Corran, Green, and Straub (12) identify with the so-called "coenzyme factor". Simultaneously von Euler's laboratory (18) has apparently come to the conclusion that their "diaphorase" is also a flavoprotein. In a preliminary report Adler, von Euler, and Günther (2) now claim to have evidence for the existence in various animal tissues of two flavoproteins, one of which they call diaphorase II which acts in a manner similar to the new yeast yellow enzyme described above to bring about the oxidation of triphosphopyridine nucleotide, and the other, diaphorase I which acts in an analogous manner for systems in which diphosphopyridine nucleotide is concerned.

The heart muscle flavoprotein isolated by Straub (40) differs in several respects from the yeast flavoproteins described above. Straub believes its prosthetic group to be flavin adenine dinucleotide because it is able to function as the coenzyme of the d-amino acid oxidase. If this is the case, then the change in the absorption spectrum of the flavin adenine dinucleotide produced by its combination with this protein is somewhat different from that encountered with the yeast flavoproteins (see Fig. 4). In the case of the yeast flavoproteins any shift encountered in the absorption spectra is towards longer wavelengths, whereas in the heart muscle flavoprotein a shift towards shorter wavelengths is found. The heart muscle flavoprotein is also unusual in that its solutions fluoresce as strongly as the free prosthetic group which, as Straub points out, suggests that no linkage involving the isoalloxazine ring is present. This flavoprotein also appears to be very stable towards heat. On the basis of its flavin content a molecular weight of about 70,000 can be calculated which

is similar to that calculated for the yeast flavoproteins on the same basis.

Like the new yeast yellow enzyme, the reduced form of this flavoprotein appears to react very slowly with oxygen. Corran et al. (12) in testing its activity made use of methylene blue in their systems in a manner similar to that described above for the new yellow enzyme. It is, therefore, not known how the reduced heart flavoprotein is reoxidized in the living cell though the participation of cytochromes-a and -b has been suggested. This flavoprotein appears to be capable of functioning with systems containing either diphosphopyridine nucleotide or triphosphopyridine nucleotide.

Another flavoprotein capable of acting as a reduced pyridine nucleotide dehydrogenase has been obtained from milk by Corran and Green (11). It appears, however, to be only about 1/10th as efficient as the heart muscle flavoprotein when tested under similiar circumstances. These authors believe that the catalytic action of this flavoprotein is not due to its reversible oxidation and reduction and suggest another role which, however, is as yet unsupported by experimental fact. This flavoprotein will be discussed further when the case of xanthine oxidase is considered.

It is thus apparent that at the present time we may have the following flavoproteins: old yeast yellow enzyme, new yeast yellow enzyme, heart muscle flavoprotein, diaphorase 1 and 11, and milk flavoprotein taking part in the oxidations of the reduced pyridine nucleotides. Whether some of these flavoproteins are identical and whether a different flavoprotein is required for each of the pyridine nucleotides are matters that can only be determined when more data are available. We may, therefore, represent the situation in a schematic fashion as shown in Fig. 5. In this diagram the substances which are enclosed in solid blocks form oxidation-reduction systems whose potentials at pH 7.0 are at the levels indicated. As represented here a substrate in the presence of its specific protein reacts with whichever of the pyridine nucleotides that functions in the reaction as coenzyme. The pyridine nucleotide is reduced and then reacts with a flavoprotein which may or may not be the same for each pyridine nucleotide. Beyond this point in the chain we cannot go with certainty. The direct reaction of the reduced flavoprotein that is formed with oxygen appears unlikely. The cytochrome system may be the next link in the chain. If this is the case, then one might expect that the reaction will proceed through the cytochromes in the order, b, c, and a, since the oxidation-reduction potentials of their systems appear to bear this relation (5).

If the flavoproteins do react through the cytochromes, then we have the interesting situation in
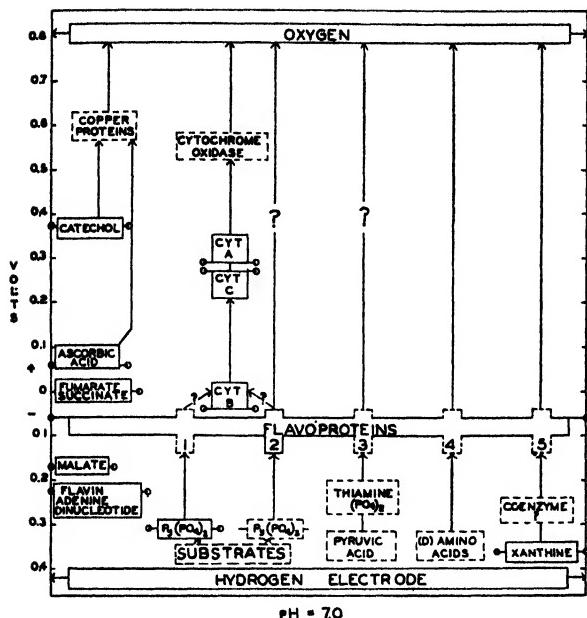
Fig. 5. A schematic representation of the role of flavoproteins in biological oxidations. Substances which are enclosed in solid blocks form oxidation-reduction systems whose potentials at pH 7.0 are at the levels indicated. Most values given are for 30° C. The sources of these data are as follows: catechol, Ball and Chen (3); ascorbic acid, Ball (4); fumarate-succinate, Borsook and Schott (9), Lehmann (31); malate, Laki (30); flavin adenine dinucleotide, Ball (8); cytochromes a, b, and c, Ball (5); flavoprotein, Kuhn and Boulanger (28); diphosphopyridine nucleotide, Clark (10), Green and Dewan (23); xanthine, Green (22), Filitti (19).

Py(PO$_4$)$_2$ = diphosphopyridine nucleotide
Py(PO$_4$)$_3$ = triphosphopyridine nucleotide.

which the flavoproteins act as a mediator between a two electron oxidation-reduction system, the pyridine nucleotides, on the one side, and a one electron system, the cytochromes, on the other. Here then, since the flavoproteins belong also to a two electron system, we have a position where a two step one electron transfer may play an important biological role by permitting an equivalence change to occur as required by the principle outlined by Shaffer (38). That the flavoproteins are suited for such a role is indicated by the observations of Michaelis and Schwarzenbach (34) that the free flavins show a two step change in the biological pH range and the observation by Haas (24) that the old yellow enzyme may form colored intermediate complexes when reacting with reduced triphosphopyridine nucleotide.

There is one other point that should be mentioned before we leave the role of the flavoproteins as reduced pyridine nucleotide dehydrogenases and that is the possible participation of the fumarate-succinate system in the chain. Szent-Györgyi (41) has suggested that a flavoprotein may act as

a mediator between the succinate-fumarate system on the one hand and the malate-oxaloacetate system on the other. This latter system he believes may in turn be a connecting link with various substrates. The potentials of these systems, as may be seen from Fig. 5, are so related that such a reaction is possible. However, to ascribe such a role to the malate-oxaloacetate system in the oxidation of substrates which require the pyridine nucleotides appears to be pointless because a pyridine nucleotide itself acts as a coenzyme for this system. In other words, the inclusion of the malate-oxaloacetate system leaves us where we started, with a reduced pyridine nucleotide to be oxidized. On the other hand, the participation of the succinate-fumarate system seems more probable. Fischer, Roedig, and Rauch (21) have recently announced that an enzyme which they call fumarate hydrase is a flavoprotein. This yeast enzyme which apparently contains flavin adenine dinucleotide as a prosthetic group is capable, according to Fischer and Eysenbach (20), of catalyzing the reduction of fumarate to succinate by suitable leuco dyestuffs. It does not, however, catalyze the oxidation of succinate and, therefore, is not identical with the enzyme known as succinic dehydrogenase. At first glance such a mode of action seems quite contrary to our usual views of an enzymatic reaction. However, is it not possible that fumarate may be capable of being reduced by a more negative flavoprotein system while the oxidation of succinate by the same system would proceed very slowly, if at all, because of the unfavorable potential relationships of the two systems? We may thus visualize as a possible cycle the oxidation of reduced flavoprotein by fumarate and the reoxidation of the succinate formed by means of succinic dehydrogenase and the cytochrome system. Such a scheme is, however, difficult to reconcile with the findings of Potter (37) who, using a heart muscle preparation containing flavoprotein, the cytochrome system, and succinic dehydrogenase among other constituents, was able to show that the oxygen uptake of the reduced pyridine nucleotides in the presence of this preparation was unaffected by factors which inhibited the oxygen consumption when succinate was the substrate.

It may be mentioned in passing that Kubowitz (27) has shown that reoxidation of the reduced diphosphopyridine nucleotide by oxygen may occur by a pathway not involving flavoprotein. The system he employed was a copper protein compound, polyphenol oxidase, and catechol. Conceivably the ascorbic acid system may also play a similar role. Such systems appear to be encountered, however, only in plant tissues and their role in animal tissues is open to question.

We must, therefore, say that we can follow the

chain of events in the oxidation of substrates by means of the pyridine nucleotides as far as the flavoprotein constituents. On the other side we can follow the participation of oxygen through the cytochrome system. At this point our knowledge of the pathway stops and a fertile field for future work lies in bridging this gap.

Pyruvate Oxidation

Another role played by flavoprotein appears to be in the oxidation of pyruvate. Here, according to Lipmann (32) in an enzyme system obtained from *B. Delbrückii,* the type of reaction may be represented as follows:

part, as is to be expected, confers on the flavoprotein its specificity.

The mode of action of this flavoprotein in the oxidation of amino acids appears to be that of a cyclic catalyst. It is reduced by the amino acid and in turn is oxidized by oxygen. The rate of reoxidation of its reduced form by oxygen appears to be greater than that of any other known flavoprotein. This would imply that the type of protein bound to the flavin adenine dinucleotide can determine not only the substrate oxidized but also the rate of reaction of the reduced form with oxygen. Negelein and Brömel (35) have compared the affinity of the oxidized prosthetic group for

$$
\begin{array}{c}
\text{Pyruvate} + \text{thiamine (PO}_4)_2 \xrightarrow{\text{protein}} \text{H}_2 \text{ thiamine (PO}_4)_2 + \text{acetate} + \text{CO}_2 \\
\uparrow \qquad\qquad\qquad\qquad \downarrow \\
\text{H}_2 \text{ flavoprotein} + \text{thiamine (PO}_4)_2 \longleftarrow \text{H}_2 \text{ thiamine (PO}_4)_2 + \text{flavoprotein} \\
\downarrow \qquad\qquad\qquad\qquad\qquad\qquad \uparrow \\
\text{H}_2 \text{ flavoprotein} \quad + \quad \text{O}_2 \longrightarrow \text{H}_2\text{O}_2 \quad + \quad \text{flavoprotein}
\end{array}
$$

Here the flavoprotein is apparently acting with diphosphothiamine in a manner similar to that described for the case of the pyridine nucleotide systems. The flavoprotein concerned in this reaction has not yet been isolated. The evidence that a flavoprotein participates is based upon the fact that an enzyme preparation inactivated by acidification and precipitation in an ammonium sulfate solution can only be reactivated by the addition of both diphosphothiamine and flavin adenine dinucleotide. Conclusive evidence for the reduction of diphosphothiamine in the process is still lacking, as is also the reoxidation of the reduced flavoprotein directly by oxygen.

Amino Acid Oxidase

The isolation of the prosthetic group of the enzyme discovered by Krebs (26), which brings about the oxidation of *d*-amino acids, led Warburg and Christian (45) to the discovery of the flavin adenine dinucleotide. Straub (39) independently reached the conclusion that some flavin compound was the coenzyme that Das (13) had earlier shown to be part of the enzyme system. The composition and some of the properties of this dinucleotide have been discussed earlier.

Though the prosthetic group of amino acid oxidase may be substituted by the prosthetic group of other flavoproteins such as the new yeast yellow enzyme, xanthine oxidase, milk flavoprotein, and heart muscle flavoprotein, the protein part is not identical with any of these. This is shown by the fact that the amino acid oxidase is unable to bring about the oxidation of the reduced pyridine nucleotides or of hypoxanthine. The protein

the protein part of the amino acid oxidase with that exhibited by the yeast yellow enzyme proteins and find it to be much lower in the case of the amino acid oxidase. On the other hand this affinity is still 40 to 400 times greater than that exhibited by the pyridine nucleotides for their protein counterparts. They have also presented evidence to indicate that in the case of the amino acid oxidase the reduced prosthetic group may be more tightly bound than the oxidized form. They found that alanine, in the presence of a small amount of flavoprotein, was unable to reduce the free prosthetic group under anaerobic conditions. Alanine was able to reduce the dinucleotide only when it was bound to the protein, and the bound reduced prosthetic group which was formed could apparently not be exchanged at an appreciable rate for the oxidized form. The reduction of the prosthetic group directly by the reduced flavoprotein apparently does not occur, which indicates that here also the flavoprotein forms a more positive oxidation-reduction system than the free prosthetic group. The tighter union of the reduced prosthetic group with the protein would be a contributing factor to this end. These workers have also obtained the protein part of the amino acid oxidase in a state which they estimate as 70 p.c. pure. On this basis and from its flavin content they calculate its molecular weight to be about 70,000.

We have, therefore, in the case of this amino acid oxidase the simplest type of system in which a flavoprotein plays a part. It is reduced directly by the substrate and is reoxidized directly by oxygen. It must be remembered, however, that the

substrate appears to be a non-physiological one and, therefore, the elucidation of the significance of this enzyme remains for the future.

Xanthine Oxidase

The other substrates whose oxidation appears to be dependent on a flavoprotein are hypoxanthine, xanthine, and aldehydes. One and the same enzyme seems to be involved in the oxidation of all three of these substrates. Ball (6) has obtained from milk a flavoprotein preparation capable of catalyzing the oxygen consumption by these three substrates, which is 500 times more active per unit of dry weight than the starting material. There can be obtained from this flavoprotein preparation two prosthetic groups. One appears to be identical with flavin adenine dinucleotide as shown by similarities in absorption spectra, activity as the coenzyme of the amino acid oxidase, and a phosphate-flavin ratio of 2 to 1. The other appears to have somewhat similar chemical properties but is colorless (8). Mixtures of the two prosthetic groups are definitely active as the coenzyme of xanthine oxidase. The flavin prosthetic group alone is, however, inactive. Whether the colorless group alone is inactive cannot be stated at the present time. The chief handicap to the clear elucidation of the possible roles of these two prosthetic groups is the fact that complete separation of the enzyme into its constituent parts without destruction of the protein is difficult. Fairly long dialysis is the best method as yet available and even then some activity still remains and flavin is still visibly present. Attempts to apply the method of acidification in ammonium sulfate solution for the separation of the prosthetic groups and the protein leads to complete destruction of the protein if sufficient acid is added to split off all the flavin. At the same time a perceptible odor of H_2S is generated. An additional complication is the fact that the free protein appears to be much more unstable than the combined form. The colorless prosthetic group appears to be the limiting factor in the reaction and it is possible that the flavin group may turn out to be unnecessary. This cannot be subjected to test, however, until a flavin-free protein preparation has been obtained.

These facts suggest two possibilities. First, the flavoprotein is a gross impurity in the preparation. However, attempts to purify the enzyme preparation further have so far been unsuccessful. If the enzyme preparation is subjected to electrical cataphoresis at different pH values or to ultracentrifugation no separation of activity and flavoprotein content can be accomplished. Moreover the flavin content of the purest preparations is of the same order of magnitude as that reported for other flavoproteins. Second, it is possible that we may be dealing with two proteins with similar properties but different prosthetic groups or one protein with two prosthetic groups. In either case the colorless prosthetic group may be looked upon as reacting with the substrate in a manner similar to the pyridine nucleotides. The flavin unit then acts as a mediator between the first prosthetic group and oxygen as indicated in Fig. 5. I can, unfortunately, tell you little about the nature of the colorless prosthetic group at the present time. It is definitely not diphosphothiamine and does not appear to be either of the pyridine nucleotides though it is present in tissues rich in these substances.

The flavoprotein itself is an interesting compound because it is markedly different in its absorption spectra from the other flavoproteins, as mentioned earlier. If the flavoprotein is treated with hypoxanthine or $Na_2S_2O_4$ in the absence of air, then a diminution of color ensues which can be restored by the admission of air. When the absorption spectrum of this reduced form is subtracted from the oxidized form then a spectrum is obtained which resembles more nearly those of other flavoproteins. Such a spectrum is shown in Fig. 4 where it is labeled "OXID-RED". The minimum molecular weight of this flavoprotein calculated from its flavin content appears to be about 74,000 and its isoelectric point is at pH 6.2. Preliminary runs with the ultracentrifuge (courtesy of Dr. Rosenfeld) using hemoglobin for comparison indicate that its molecular weight is as great as, as if not greater than, 68,000.

Corran and Green (11), by a different procedure than that used by Ball, have obtained from milk a flavoprotein whose absorption spectra for that range in the visible which was determined is similar to that for the xanthine oxidase preparation (see Fig. 4). They give, however, a value for the height of its absorption peak at 279 mμ which is about 1/3 that encountered in this region for other flavoproteins. Their preparation was described as being devoid of xanthine oxidase activity though it did function as a reduced pyridine nucleotide dehydrogenase. The molecular weight of this flavoprotein as determined by Philpot (36) by means of the ultracentrifuge was 220,000. Since the preparation used was not pure he suggests that the molecule contains 8 flavin groups and has a molecular weight of 270,000 to 320,000. Dixon (15) in a recent review article states (without giving a reference) that Corran and Green have now obtained a flavoprotein preparation from milk which shows xanthine oxidase activity but which is not reduced anaerobically by hypoxanthine. It is thus difficult at present to decide what relationship these two flavoprotein

preparations from milk bear to one another. I am inclined to believe, however, that the two preparations will eventually be found identical and that the xanthine oxidase activity of the preparation depends upon whether the colorless prosthetic group has remained intact during the isolation procedure.

Conclusion

It is thus apparent that flavoproteins play an important role as mediators in a wide variety of biological oxidations. A large number of flavoproteins are now known to exist whose prosthetic groups appear to be identical. Further work is, however, needed to establish this for a fact. In the case of some of the flavoproteins there is no doubt that the protein parts are not identical and that specificity of action is thereby conferred. In other cases isolation and comparison of properties has not yet advanced sufficiently to permit a generalization. This is especially true of those flavoproteins which bring about the reoxidation of the two reduced pyridine nucleotides and reduced diphosphothiamine. Whether one and the same flavoprotein may be reduced by the somewhat similar grouping possessed by these three compounds and how it is then reoxidized in the living cell will be questions whose answers will undoubtedly be energetically sought for in the coming year.

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DISCUSSION

Dr. Barron: I would like to ask Ball's opinion about the possible existence of biological oxidations where the alloxazins act directly as the electron transfers to molecular oxygen. So far, the oxidation enzymes which have been isolated are entirely artificial systems. Let us take the system which oxidizes hexose monophosphate to

phosphohexonic acid, where alloxazins transfer electrons directly to molecular oxygen. The activating protein of this system was prepared from red cells. We know that red cells do not oxidize hexose monophosphate. On the other hand I have been able to find some bacteria (soil bacteria) which oxidize hexose monophosphate to phosphohexonic acid as in the preceding case. Here, the oxidation is completely inhibited by HCN, an indication that a heavy metal catalyst is part of the enzyme system.

Now we may go further and take the system d-amino acid oxidase. As Ball has pointed out, the oxidation system is made up of an activating protein and alloxazin dinucleotide. The oxidation of d-amino acids by this system is not inhibited by hydrocyanic acid. A number of bacteria are able to oxidize d-amino acids; the oxidation is completely inhibited by hydrocyanic acid. So here too we cannot find a direct oxidation from alloxazin to molecular oxygen. If you go over the literature, it is difficult to find one naturally existing enzymic system where electron transfer goes directly from alloxazin to molecular oxygen.

The experiments of Lipmann on the enzyme components for the oxidation of pyruvic acid also do not agree with experiments in natural oxidation systems containing bacteria or animal tissues. We must point out that Lipmann has been working with an enzyme preparation which was found by Davies, and obtained from B. Delbruckii, a bacterium which is completely insensitive to HCN.

As Warburg pointed out, the respiration in anaerobic bacteria may be carried through the mediation of alloxazins. So I am inclined to think that Lipmann has unfortunately chosen a system where the path of oxidation is exceptional and has nothing to do with the path of oxidation of pyruvic acid in aerobic cells. I say this because we must be on our guard against making a generalization from enzyme systems. The danger of such generalization can be shown in the oxidation of lactic acid. As Ball pointed out, lactic acid in muscle is catalyzed through the mediation of pyridine nucleotide, alloxazin dinucleotide and the cytochrome system.

In gonococci the dinucleotide and alloxazin systems disappear. The lactic acid is directly oxidized through the cytochrome system. Finally if one takes anaerobic bacteria like Streptococcus hemolyticus, the oxidation does not go through the cytochrome system because in these bacteria the oxidation of lactic acid is insensitive to HCN. So here we have one substrate, lactic acid, being oxidized in three different ways.

Dr. Ball: I am in entire accord when Barron says that substrates may be oxidized through other pathways than that involving a flavoprotein. As to whether the reduced flavoproteins are directly oxidized by oxygen in living cells, we certainly cannot give a general answer. For example, as I pointed out, we know very little even about how the reduced flavoprotein reacts with oxygen in isolated systems where the pyridine nucleotides are concerned. Certainly, however, a direct reaction with oxygen is not involved in these cases. On the other hand, in the isolated xanthine oxidase system, where the reaction does take place directly with oxygen, I have attempted without success to increase the rate of oxygen consumption by adding a heart muscle preparation which is active in the pyridine nucleotide system. If methylene blue is added you can about double the rate of oxygen uptake in the xanthine oxidase system. I may add that it is well known that all biological oxidations involving oxygen apparently are at least 90 per cent inhibited by cyanide, which is further evidence that direct reaction of flavoprotein with oxygen is not important to the bulk of respiration.

Dr. Burk: Is there anything that you can say about the actual rates of oxygen oxidations carried out by flavoproteins as compared with other enzymes that we know react with oxygen (Warburg-Keilin system)? It is not clear in my mind just how fast the flavin compounds are now believed to react with oxygen. It is my understanding that the earlier compounds reacted comparatively slowly.

Dr. Ball: I can give no concrete answer as to the relative rates of reoxidation of the reduced flavoproteins by oxygen as compared to other autoxidizable substances. All I can say is that the reduced form of the amino acid oxidase appears to react very rapidly with oxygen, while the reduced new yeast yellow enzyme seems to react very slowly.

Dr. Michaelis: Might it not be possible that the sluggishness of reactivity of the reduced flavins with oxygen is analogous to the same sluggishness of other leuco dyes, with the difference that the pH range in which this sluggishness occurs is greater for the flavins? Has it ever been investigated whether traces of copper (in the form of some loosely bound copper complex) play any role in the autoxidation of the flavins?

Dr. Ball: I know of no such investigations.

Dr. Barker: Since the prosthetic group of so many of these flavoproteins seems to be the same, is there any evidence as to what it is in the protein that regulates the activity of flavoprotein itself?

Dr. Ball: That is a very interesting question, but I think we will have to wait a great number of years for the answer. I would like to point out, however, that we must not be too willing as yet to say that the prosthetic group of all these flavoproteins is identical. In most cases the evidence that the prosthetic group is

identical with flavin-adenine-dinucleotide is very slim. Only in the case of the amino acid oxidase has careful isolation of the prosthetic group been performed, and complete chemical analysis made.

Dr. Stotz: You said we are interested in the gap from the flavoprotein up to the cytochrome system. I understand that with the two pure constituents, cytochrome-c and the Haas flavoprotein, there is no direct relation between what we know about the pure prosthetic groups, the flavins themselves, with cytochrome-c. We are looking for something on the order of methylene blue that can act as a mediator. Have you any suggestions about such a compound? Could it possibly be a free flavin? Cytochrome-b has been suggested. I wonder if we could guess a little as to what such a substance might be?

Dr. Ball: The only answer I can give to your questions is this fact. The oxidation-reduction potential of the free flavin is about 140 mv. lower than that of the flavoprotein system. Therefore such a role as you mention for the free prosthetic group does not seem probable.

Dr. Korr: I should like to ask a question in connection with the comments of Barron and the question of Burk. Can you reconcile the apparent non-autoxidizability of reduced flavoprotein *in vivo* with its ready oxidation by oxygen *in vitro*? I am thinking particularly of some observations of Ogston and Green. They showed that the oxidation of hexose mono- and diphosphate by oxygen, in the presence of their specific dehydrogenases, was markedly catalyzed by flavoprotein; in intact yeast, their oxidation was completely inhibited by HCN. One would expect, on the basis of the *in vitro* observation, that after poisoning the cytochrome-oxidase system with HCN the oxidation of these substrates would proceed to at least an appreciable extent through the intracellular flavoprotein.

Dr. Ball: We have been discussing for the most part isolated systems in which we can control the amount of components employed. In living tissues we must take components in the concentrations in which we find them and in such cases it is an open question as to whether you are going to find sufficient flavoprotein for the oxidation to proceed directly with oxygen at a measurable rate.

Dr. Shorr: As one views the ever increasing numbers of flavoproteins, one is tempted to consider the possibility of simplifying mechanisms, by which it would not be necessary for all of them to be present in the cell at one time. Is there any experimental evidence to indicate that the prosthetic groups can change from one protein to another in response to the necessity to deal with one type of substrate or another? Or must we consider them to be rigidly fixed and prepared to deal with a wide variety of substrates at all times?

I raise this point because of its implication in connection with some work which we have been carrying out in my laboratory with diabetic tissue. Such tissue when first removed from the diabetic organism lacks the capacity to oxidize carbohydrates, and deals with lactic and pyruvic acid in different fashion from similar tissues from normal animals. It is reasonable to assume defects in the enzyme systems as responsible for these deficiencies. Yet, upon incubation of these tissues at body temperatures in phosphate-Ringer solutions for 8-10 hours, there is a gradual restoration of carbohydrate metabolism until the respiratory quotient indicates that complete carbohydrate metabolism is taking place. We must assume, as an explanation for this change, either a resynthesis in the cell of the enzyme systems which control carbohydrate oxidation, or the release of some type of inhibition. If the former explanation should be found to hold, it would offer a simplifying mechanism which might be operating for other systems as well.

Dr. Ball: I would consider the exchange of prosthetic groups from one protein to another as possible. I would expect such an exchange, however, to be governed by the relative affinities of the proteins concerned with the prosthetic group rather than by the type of substrate present. It is, however, conceivable that the substrate may influence the affinity of the protein for its prosthetic group, although I know of no evidence to support such an idea.

Dr. Abramson: Have anti-bodies to these flavoproteins been produced?

Dr. Ball: Not to my knowledge.

Dr. Baumberger: Is it possible that the protein may be modified in the course of anaerobiosis so that relative concentrations of yellow enzymes of particular types will be different and thus change the course of oxidation?

Dr. Ball: It is conceivable, I believe, that oxidation-reduction changes in the protein molecule itself may influence its ability to link with the prosthetic group, especially if a sulfhydryl group is directly concerned in the linkage. I know, however, of no evidence to support such an idea. I mentioned the fact that when a xanthine oxidase preparation was treated with acid and the flavin group split off, an odor of H_2S was also generated. Whether this signifies the participation of a sulfhydryl group in the linkage in this case cannot be decided without further data.

We know very little in general about the relative affinity of the oxidized and reduced prosthetic groups for their respective proteins. In amino acid oxidase, however, the reduced prosthetic group seems to be bound more tightly than the oxidized form.

CYTOCHROME OXIDASE AND CYTOCHROME

Elmer Stotz

Since there appear to be at least four papers to be presented in this Symposium which must, more or less directly, be concerned with the subject of cytochrome oxidase and cytochrome, I have attempted in half of this paper only to briefly describe the subject, and in the second half to describe in more detail the results of personal researches. In this way I hope the later papers will amplify my introduction and possibly avoid undue repetition.

Although as early as 1887, MacMunn (1) apparently observed and separated from muscle the substance now known as cytochrome-c, the interest in iron as a cellular respiratory catalyst was aroused only in 1924 by Warburg (2). Upon observing the cyanide sensitivity of cellular respiration in conjunction with the catalytic behaviour of the hemin-charcoal model toward certain organic substances, Warburg gave the name *"Atmungsferment"* to the catalytically active iron compounds involved in cellular respiration. It was postulated that this substance (or group of substances) could in turn be reduced by organic substances of the cell and oxidized by molecular oxygen.

Shortly thereafter, Keilin (3, 4) observed spectroscopically in bacteria, yeast and animal tissues, a hematin pigment composed of three components, called by him cytochromes-a, -b, and -c. The bands were readily observed in layers of tissue or in suspensions of cells only under anaerobic conditions or upon addition of reducing agents. Fig. 1 illustrates the position of these bands. The band at 6050 A is the a-band of cytochrome-a. It is a broad band and is not homogeneous in different sources, varying from 5950-6150 A. Recent work shows, in fact, that it is due to more than one substance (5, 6). The a-band of cytochrome-b lies at approximately 5650 A and is somewhat more homogeneous and constant in position than that of cytochrome-a. The sharpest and most easily observed band, however, is that of cytochrome-c, lying at 5500 A. The complex band at 5200 A is due to the β-bands of cytochromes-b and -c, the β-bands of cytochrome-a probably being obscured in the region of the strong bands of cytochromes-b
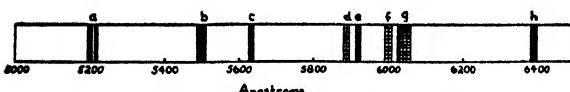
and -c. These bands disappear under aerobic conditions and give rise to very weak bands of the oxidized cytochromes, but reappear unchanged upon again establishing anaerobiosis. One or more of the cytochromes may be lacking in certain organisms, but in animal tissue all three are generally present.

Meanwhile in Warburg's laboratory very careful studies concerning the inhibition of yeast respiration by carbon monoxide and its reversibility by light was in progress (7, 8). By measuring the reversible effect of light at various frequencies and measured intensities on the CO-inhibited respiration of yeast, the work culminated in the construction of the relative CO spectrum and later the absolute CO spectrum of the *Atmungsferment* (9, 10). It was renamed the "oxygen-carrying enzyme." The spectrum obtained was obviously that of a hemin-containing compound, and in type resembled that of *Spirographis* hemin (11). The *Spirographis* porphyrin has been found by Fischer and Seeman (12) to differ from ordinary protoporphyrin in that one of the vinyl groups is oxidized. It is therefore likely that the oxygen-carrying enzyme of Warburg also contains such an oxidized hemin, and may therefore be classified as a pheohemin-protein compound which either combines with or is oxidized by oxygen and unites with carbon monoxide reversibly. The a-band of the carbon monoxide complex lies at 5920 A and the γ-band at 4520 A.

In 1928, however, Keilin (13) investigated the properties of an enzyme in yeast and heart muscle which he believed identical with that described by Warburg. This was called "indophenol oxidase" since it could be detected by the oxidation of leuco-indophenol, likewise of *p*-phenylene diamine. Employing this test, Keilin demonstrated that the oxidase was destroyed at 70° C., was inhibited by KCN and H_2S and irreversibly destroyed by drying or acetone treatment. The oxidation of *p*-phenylene diamine was slow unless its simultaneous reduction by dehydrogenase systems was prevented by agents such as narcotics or mild heating. It was further found that this oxidation was inhibited by CO, and that the inhibition was reversed by light. The partition constant K was approximately 10, which was the value found by Warburg for the oxygen-carrying enzyme.[1] These observations were valuable in demonstrating that the oxidase could be investi-

Fig. 1. Diagrammatic representation of the spectra of the cytochromes and the respiratory enzyme. a, β-bands of cytochromes -b and -c; b, a-band of cytochrome-c; c, a-band of cytochrome-b; d, oxygen-carrying enzyme (?); e, CO compound of oxygen-carrying enzyme (cytochrome-a_3); f, a-band of cytochrome-a_3; g, a-band of cytochrome-a; h, CN compound (oxidase?).

[1] $K = (n/1-n) \cdot (CO/O_2)$ where n is the O_2 consumption in the $CO + O_2$ gas mixture represented by the ratio CO/O_2 and $1-n$ the difference in the O_2 consumptions in gas mixtures containing N_2 and CO, respectively, where $N_2/O_2 = CO/O_2$.

gated using a simple substrate such as *p*-phenylene diamine and in a homogeneous solution, rather than as a single component of a more complicated system in the intact cell.

Keilin extended this work by studying the factors which affected the oxidation and reduction of the three cytochrome components in yeast and in crude heart muscle extract. He demonstrated clearly that those factors (KCN, H_2S, etc.) which inhibited the oxidase thereby retarded the oxidation of the cytochromes in the extract, while those which affected the reducing mechanisms (dehydrogenases) retarded the reduction of the cytochrome components. Cytochromes-a and -c were found to be non-autoxidizable, while the -b component reacted with oxygen directly. The joint action of isolated yeast cytochrome-c and oxidase of heart muscle was further demonstrated manometrically using cysteine as a reducing agent for the cytochrome. A similar experiment was demonstrated by Stotz and co-workers (14) using ascorbic acid as a substrate. Evidently cytochrome and its oxidase function together as a single autoxidizable substance. A reversible system such as methylene blue can replace the natural cytochrome system, for example, in the oxidation of succinate. Other artificial dyes can also replace this system and their efficiency as catalysts depends on their oxidation-reduction potential (15). Warburg and co-workers did not agree, however, that "indophenol oxidase" was identical with the oxygen-carrying enzyme. A paper in 1933 by Warburg, Negelein and Haas (16) sets forth their position clearly. A scheme was presented whereby there are 5 different compounds undergoing $Fe^{++} \rightarrow Fe^{+++}$ changes, the first representing the oxygen-carrying enzyme, the last three the cytochromes. The second is an iron compound whose function is to reduce the oxygen-carrying enzyme and oxidize the cytochromes (which might therefore be Keilin's "indophenol oxidase"). They state that only the oxygen-carrying enzyme is very rapidly autoxidizable, explaining the fact that, at least in certain bacteria, the respiration is independent of the oxygen tension down to very small pressures (17). Since, when the respiration is partially inhibited by carbon monoxide, it is then dependent on the oxygen tension (8), carbon monoxide must combine with the ferrous form of the enzyme and prevent its autoxidation. Lastly, cyanide inhibition is independent of the O_2 tension (8), hence cyanide must inhibit only the reduction of the enzyme. In the highly respiring *B. Pasteurianum* under anaerobic conditions, a weak band was observed at 5890 A which was attributed by Warburg and Negelein (18) to the ferrous form of the oxygen-carrying enzyme since it was shifted to approximately 5920 A by addition of carbon monoxide. The latter is the posi-

tion predicted for the carbon monoxide compound by the photochemical methods already mentioned. Upon addition of cyanide a new band appears at 6390 A. This could not be attributed solely to the cyanide complex of the oxygen-carrying enzyme since under certain conditions the complete band of the reduced enzyme (5890 A) could be observed simultaneously. Tamiya and Kubo (19) gave experimental support to a theory that carbon monoxide affects only the oxygen-carrying enzyme, whereas cyanide inhibits the oxidase, that is, two factors are present. The cytochromes themselves were apparently not affected by either carbon monoxide or cyanide.

Up to 1933 Keilin (20) maintained, however, that the oxygen-carrying ferment and "indophenol oxidase" were identical, and that the 5890 A band was probably a degradation product of cytochrome-a components. It appears true that the 5890 A band is observed only when the cytochrome-a band is lacking (21), but this may be due simply to the practical difficulty in observing such a weak band in the presence of neighboring strong absorption. Bacteria lacking in cytochrome-a sometimes show this band at 5890 A (also called cytochrome-a_1) and a band at 6300 A (called cytochrome-a_2.).

An interesting paper from Keilin's laboratory has appeared very recently (22), which reports a more careful spectroscopic study of heart muscle extract. It was observed that the cytochrome-a band did not behave as a unit, but that the portion lying at 6000 A (a-band) was associated with a γ-band lying at 4480 A. The substance responsible for these bands was autoxidizable and was called cytochrome-a_3. In the presence of carbon monoxide, the a-band of cytochrome-a_3 shifts to 5920 A and the γ-band to 4520 A. Cyanide, H_2S, NaN_3 and NH_2OH (which are typical "oxidase" inhibitors) unite with the a_3 component and prevent formation of its carbon monoxide complex. The position of the a and γ bands of the CO compound coincide exactly with the photochemical absorption spectrum of Warburg. It was concluded therefore that cytochrome-a_3 is identical with the oxygen-carrying enzyme. This would seem to identify the oxygen-carrying enzyme, but there may be dispute over the position of the reduced ferment itself, since Warburg claimed that it was at 5890 A and Keilin at 6000 A.

Since cytochrome-a_3 was also affected by "oxidase" poisons, it seemed obvious that this was also the "oxidase". Keilin himself, however, offered very real arguments against this: (*1*) Oxidation of the three cytochrome components was possible in spite of the CO band of cytochrome-a_3 being visible, indicating that cytochrome-a_3 was not responsible for oxidation of the cytochromes; (*2*) upon adding reduced cytochrome-c to the

heart muscle extract, cytochrome-a_3 was not reduced; (3) no change in the CO spectrum could be observed upon irradiation with light; and (4) certain bacteria which are devoid of the cytochrome-a complex are nevertheless affected by CO and cyanide. Certainly the picture is not yet clear; it is very possible that with the many hemin complexes of similar spectra and chemical properties which may exist in tissue extracts, the spectroscopic observations on such materials may never explain the situation, and isolation of the several components must be awaited.

Cytochrome-c

Of the three cytochrome components, only cytochrome-c appears to be very stable and easily extracted from tissue. Yakushiji and Mori (23) claim to have isolated cytochrome-b from yeast in an impure state, but the work has not been confirmed and is not generally accepted. Cytochrome-c, on the other hand, was extracted from yeast in 1930 by Keilin (24). In 1936 it was first prepared in the pure state by Theorell (25) and shortly thereafter by Keilin and Hartree (26) using a much simpler procedure, both employing beef heart as the source. Neither preparation has yet been crystallized, but both investigators were at that time unable to purify it further. It contained, like hemoglobin, 0.34 p.c. Fe and was apparently homogeneous electrophoretically and in the ultracentrifuge. The molecular weight was found to be 16,500. It was surprising to hear, therefore, that Theorell[2] now has produced by cataphoresis a preparation containing 0.40-0.43 p.c. Fe. Reduced cytochrome-c has an α-band at 5500 A and a β-band at 5200 A, the same as observed in a suspension of cells. It is a particularly stable hemin-protein combination, withstanding as high as 2N acid or alkali and reverting to the original spectrum and catalytic properties on neutralisation. Theorell (27) has recently investigated the structure of cytochrome-c and believes its peculiar stability is related to a double thio-ether linkage between the protein and the hemin.

Coolidge in 1932 (28) measured the oxidation-reduction potential of an impure cytochrome-c isolated from yeast. The preparation gave very unsatisfactory potentials with the electrode but the potential could be stabilised by the addition of small amounts of quinhydrone. By adding oxidant or reductants to a point where the cytochrome spectrum just changed, and then recording the potential, he gave a value of $E'_o = 0.260$ v. at pH 7.0 with lower values at pH 5.0. Green (29) employing standard methods of potentiometric titrations, but using impure yeast cytochrome-c,
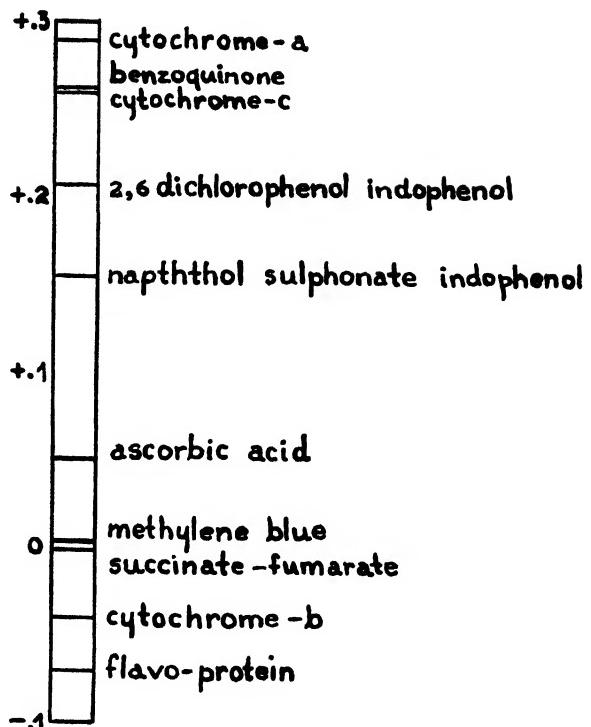
Fig. 2. Oxidation-reduction potentials (E'_o) at pH 7.2 of the cytochromes and other systems.

recorded a very different value, namely $E'_o = 0.127$ v. between pH 4.6 and 7.1.

After the technique of purification of cytochrome-c was announced, it was not surprising to find the discrepancies in these figures quickly corrected. Wurmser (30) using a potentiometric method obtained a value of $E'_o = 0.254$ v. and Stotz, Sidwell and Hogness (31) obtained a value of 0.262 v. between pH 5.0 and 8.0 using a spectrophotometric method. The latter investigators were able to measure with the use of an accurate photoelectric spectrophotometer (32) the amounts of oxidized and reduced indicator and of cytochrome in equilibrium with each other. Knowing accurately the potentials of the indicators used as developed by Clark (33), the potential of the cytochrome was easily calculated by the equation:

$$E'_{oA} - E'_{oB} = (RT/F) \ln K_{eq.}$$

where E'_{oA} is the potential of the indicator, E'_{oB} the potential of the cytochrome and $K_{eq.}$ the the equilibrium constant as determined by the ratio of oxidized and reduced forms of A and B. In this method only small amounts of cytochrome were required.

Just prior to these investigations, Ball (34), using a spectroscopic method, was able to estimate the potentials of the three cytochromes as they existed in a crude heart muscle extract. By ob-

serving the intensity of the bands of the completely reduced components, then poising the potentials with definite ratios of the oxidized and reduced forms of systems such as the succinate-fumarate, ascorbic-dehydroascorbic acid, and hydroquinone-quinone, the intensities of the bands were changed. An estimate was made of these changes and from this the potentials of the cytochromes could be judged. He recorded the value of —0.04 v. for cytochrome-b, + 0.27 v. for cytochrome-c and + 0.29 v. for cytochrome-a.

These potentials, in relation to other compounds of biological interest, are shown in Fig. 2. The high potential of cytochrome-c obviously places it in a favorable position for reduction, but it is not surprising to find that it is only slowly autoxidizable and requires an oxidase for its reaction with oxygen. The position of cytochrome-b suggests that it should be an important stepping-stone in the chain of respiratory catalysts, but thus far a definite function has not been conclusively demonstrated.

Oxidase and cytochrome relations

The availability of pure cytochrome-c and the use of "oxidase" as prepared from beef heart muscle (15) has made possible a much better understanding of the relations between the oxidase and the cytochromes. It was noted, for example, that not only p-phenylene diamine, but any of the common indophenol dyes could be oxidized by heart muscle "indophenol oxidase". Although it was assumed for a long time that "indophenol oxidase" was a single substance and much time was spent in estimating its activity in tissue by the oxidation of Nadi reagent, it seemed strange that a single enzyme could be so unspecific towards substrates. In view of the high potential of cytochrome-c it was apparent that the non-specificity of the oxidase might be attributed to reduction of the electromotively active cytochrome-c in the preparations, with subsequent aerobic oxidation of the reduced cytochrome by the oxidase. In studying the oxidation of leuco dyes by oxidase preparations it was noted that the rate of oxidation in each case rapidly decreased at a pH where the potential of the dye and of cytochrome-c became equal. Obviously the oxidation of these dyes was primarily dependent on the cytochrome-c. Keilin and Hartree (35) likewise noted differences in the rate of oxidation of various substrates and the fact that added cytochrome-c accelerated all these oxidations. The latter authors therefore renamed the enzyme "cytochrome oxidase."

Stotz et al. (36) have given evidence, however, that in the oxidation of some substrates, cytochrome-b as well as cytochrome-c may be operative. If, for example, as in Fig. 3, we compare the cyanide sensitivity of the oxidation of p-pheny-
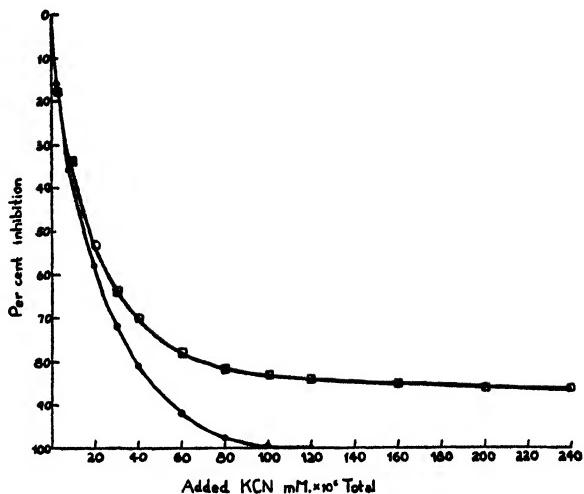
Fig. 3. Sensitivity of hydroquinone and p-phenylene diamine oxidations to cyanide. ● hydroquinone, □ p-phenylene diamine. $T = 38°$ C. pH 7.15. 15 units oxidase. 23.5×10^{-6} mM cytochrome total.

lene diamine with that of hydroquinone using a typical oxidase preparation plus cytochrome-c, we find that complete inhibition of p-phenylene diamine oxidation is not readily attained. In contrast, hydroquinone oxidation is completely inhibited by low concentrations of cyanide. When it is recalled that the potential of the hydroquinone system (at pH 7.15) is practically equal to that of cytochrome-c but much higher than that of cytochrome-b, it is logical to suppose that hydroquinone oxidation is dependent only on the cytochrome-c and oxidase. Since, however, the potential of p-phenylene diamine is lower than that of cytochrome-b, and cytochrome-b is insensitive to cyanide and autoxidizable, it is obvious that this oxidation is conditioned by cytochrome-b as well as by cytochrome-c. Judging from experiments with an oxidase preparation essentially free of both cytochromes-c and -b, it cannot be said that cytochrome-b is *essential* in the oxidation of p-phenylene diamine, for example, but this experiment does illustrate that cytochrome-b can function either independently or with cytochrome-c. It may, however, be quite essential in the oxidation of physiological reducing agents. In this connection it is of interest to find that the new flavo-adenin-protein of Haas (37) cannot reduce cytochrome-c directly, and cytochrome-b has been suggested as an intermediate.

It has already been stated that the oxidation of hydroquinone, for example, by heart muscle is dependent both on cytochrome-c and cytochrome oxidase, hence its rate of oxidation by a simple extract is neither a measure of the oxidase nor of the cytochrome. If, however, (see Fig. 4) to an oxidase preparation which in itself produces a
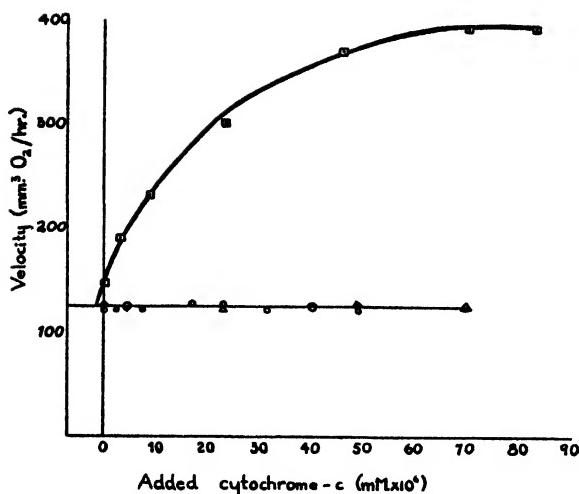
Fig. 4. Oxidation of hydroquinone by the oxidase-cytochrome system. ◯ cytochrome-c alone, △ cytochrome-c + heat-treated oxidase, □ cytochrome-c + oxidase. $T = 38°$ C. pH 7.15.

relatively slow oxidation of hydroquinone, cytochrome-c is added, a rapid oxidation is established. Cytochrome-c of itself does not accelerate the autoxidation of hydroquinone. The reduction of cytochrome-c by the hydroquinone is visibly very rapid, hence the limiting velocity in this reaction must be the oxidation of the reduced cytochrome. With any given amount of oxidase, a maximum effect of added cytochrome on the velocity of oxidation is attained, suggestive of an enzyme-substrate complex between the oxidase and cytochrome. Such curves have in fact been analyzed (38) according to Lineweaver and Burk (39) and have been found to comply with the laws of a typical enzyme-substrate complex.

Of greater interest was the fact that in the presence of an excess of cytochrome-c, the velocity of oxidation of hydroquinone (discounting its autoxidation) was directly proportional to the amount of oxidase added. This offers a method of estimating the oxidase. An arbitrary unit of oxidase was defined as the amount which, in the presence of an excess of cytochrome-c, produces an increase of 10 mm³./hr. over the autoxidation rate of hydroquinone; the pH, temperature, and concentration of hydroquinone being constant. Obviously the maximum activity of the oxidase in any preparation can only be judged in the presence of an excess of cytochrome-c. It is little wonder that according to older tests the oxidase could not be purified, since any operation freeing the preparation of cytochrome-c would have indicated to the worker a loss of oxidase activity.

Employing the newest test we have been able to prepare an oxidase practically free of cytochrome-b and -c (spectroscopically), having in itself almost no activity in oxidizing hydroquinone or p-phenylene diamine. Fig. 5 illustrates the effect of successive acetic acid precipitations and resuspensions of a heart muscle preparation. It may be recalled that in the oxidation of p-phenylene diamine by an oxidase preparation, the part due to cytochrome-c (and the oxidase) may be inhibited by cyanide while the relatively cyanide-insensitive part is probably due to cytochrome-b. If then, the effect of the same amount of oxidase (as determined with an excess of cytochrome-c) was tested on p-phenylene diamine oxidation and the effect of cyanide with these preparations studied, it was possible to estimate relatively the amounts of cytochromes-b and -c left in the preparation. The series of curves in Fig. 5 indicate that acetic acid precipitations quickly deplete the preparation of the bulk of the cytochrome-c and partially of the cytochrome-b. This was confirmed by spectroscopic observations of the enzyme suspensions. Two acetic precipitations and a long dialysis result in an active oxidase with practically no activity by itself on p-phenylene diamine. Such preparations, although weak in cytochromes-b and -c, nevertheless show a strong cytochrome-a band.

The effect of carbon monoxide and cyanide was re-studied in the presence of different amounts of cytochrome (38). Although there is evidence that carbon monoxide unites to some extent with cytochrome-c even at physiological pH (40), these experiments demonstrated that the inhibiting effect of these substances was concerned with the oxidase.

In all this work we naturally attempted to find evidence for the function of cytochrome-a and for a possible dual character of the oxidase. If cytochrome-a were essential or the oxidase had a dual nature, it should reflect on the Michaelis constant of the oxidase-cytochrome complex. With all of our preparations, however, and even upon frac-
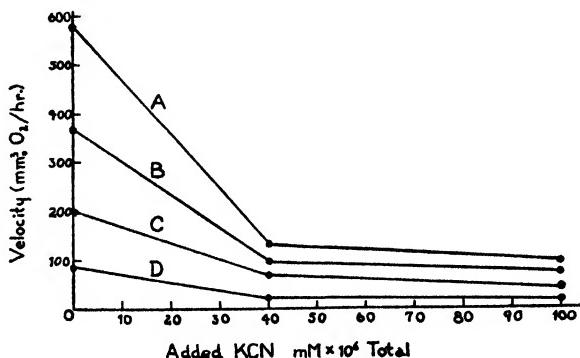
Fig. 5. The effect of successive acetic precipitations on p-phenylene diamine oxidation. A, crude extract; B one precipitation and resuspension. C, two-fold precipitation. D, three-fold precipitation. T = 38° C. pH 7.15. 20 units oxidase.

tionation by heat and acid treatments, we were unable to correlate the intensity of the cytochrome-a band with activity, or appreciably change the Michaelis constant. Our work therefore implies that cytochrome oxidase is a single substance which catalyzes the aerobic oxidation of reduced cytochrome. The mechanism involves combination with the cytochrome to form a complex, the autoxidation of which is inhibited by carbon monoxide or cyanide.

The fact that cytochrome oxidase is sensitive to carbon monoxide is not proof, however, that it is identical with Warburg's oxygen-carrying ferment. Such reactions in cell-free media and at high oxygen tensions may not require the oxygen-carrying enzyme at all; all of Warburg's experiments dealt with the intact cell. The situation might well be compared with the question of myoglobin; this is not considered necessary in an isolated system but quite essential in the intact tissue. Likewise in the case of flavoprotein, although it is autoxidizable, it is apparently oxidized chiefly by cytochrome in the complete respiratory system (41). Since it is unquestioned that the oxygen-carrying ferment is a hemin-containing enzyme, Keilin (5, 22) himself virtually admits that the two factors are different by his recent suggestion that cytochrome oxidase is a copper-protein. This suggestion was based largely on the observation of Cohen and Elvehjem (42) that in copper deficiency the oxidase content of liver is greatly decreased, and also by analogy with the copper protein (catechol oxidase) of Kubowitz (43). The fact that copper deficiency produces a decrease in liver and heart oxidase has been confirmed by Schultze (44) using the newer test for the oxidase described in this paper. He pointed out, however, that copper may only be essential for the formation of the oxidase, analagous to hemoglobin formation. Against the supposition that cytochrome oxidase is a copper-protein is the fact that typical copper inhibitors such as are effective in blocking the enzymic oxidation of catechol (43) or ascorbic acid (45) do not inhibit cytochrome oxidase.

Distribution of cytochrome oxidase and cytochrome-c

Oxidase distribution in rat tissues according to the new method described, has been measured in our laboratory (46). The ground tissue was dialyzed overnight against phosphate buffer to eliminate residual respiration of the tissue, then studied for its oxidative power on hydroquinone in the presence of an excess of cytochrome-c. The activity was expressed as arbitrary units of oxidase per mg. of nitrogen in the extract tested. In this way reasonably good agreement between

animals was obtained. More striking, however, was the fact that in spite of animal to animal variation, in individual animals there was consistent agreement in the order of activities of the various tissues. The average values of the oxidase activities converted to units of oxidase per mg. of dry tissues are shown in Table I.

The order of activities shown obviously parallels the metabolic activities of the tissues. Heart muscle is approximately 30 times as active as the least active tissue studied, namely large intestine. If the tissues are listed in the order obtained by expressing the figures as units of oxidase per mg. N instead of per mg. dry weight in the extract tested, they fall in the same order except that brain and kidney are equally active. In the column labelled Q_{O_2} are recorded a few values of the oxygen consumption of tissues. These were taken from Krebs' review (47) and represent only undisputed values confirmed by several workers. They also fall in the same order as the oxidase activities of the tissues. This demonstrates that the oxygen consumption of the tissues examined depends greatly on the oxidase system and is proportional to their oxidase contents.

A method has also been developed (48) to determine the cytochrome-c content of at least the larger tissues of the rat. In this method the tissue is ground with sand and water and extracted at pH 3.5-4.0 with trichloracetic acid. After neutralisation of the extract to eliminate further inactive protein, all of the remaining protein, including the cytochrome, is precipitated by phospho-tungstic acid. The precipitate is dissolved in dilute ammonia, and the phospho-tungstate eliminated as the barium salt. Adsorption of the cytochrome is prevented by an excess of phosphate, and a heating operation results in a clear solution containing the cytochrome. Such solutions from the several tissues examined can be tested manometrically for cytochrome-c content by their activity in conjunction with a typical heart muscle oxidase preparation. It will be recalled that the addition of cytochrome-c to an oxidase preparation produces, up to a certain point, large increases in the oxidation rate of hydroquinone. If then a "standard" curve is determined using pure cytochrome-c, unknown cytochrome solutions can be tested and the activities obtained converted into milligrams of cytochrome from the standard curve. The method was not affected by methemoglobin, metmyoglobin or flavin which might be present in the final solution to be tested; cytochrome added during the grinding stage was recovered 80-95 p.c. As little as 0.1 γ of cytochrome iron can be estimated with an accuracy of 10 p.c. by this method.

The results obtained in a series of 10 animals showed much greater consistency than in the case of the oxidase. In Table I is recorded a summary

TABLE I.

Cytochrome oxidase and cytochrome-c content of rat tissues

Oxidase (Units/mg. dry wt.)		Q_{O_2}		Cytochrome mg./gm. dry wt.)	
Heart	9.7			Heart	2.34
Kidney	4.7	Kidney	21.0	Kidney	1.36
Brain	3.5	Brain	14.0	Skel. Mus.	0.68
Skel. Mus.	2.3			Brain	0.35
Liver	1.7	Liver	9.0	Liver	0.24
Spleen	1.6			Spleen	0.21
Lung	1.3	Lung	6.0	Lung	0.14
Testis	1.1				
Dia. Mus.	0.72				
Large Intest.	0.36	Large Intest.	3.0		
Embryo				Embryo	
(3-5 wk.)	1.1			(2-4 wk.)	0.03
				(5-6 wk.)	0.18
Tumor				Tumor	
R256	2.9			R256	0.02
				R39	0.03
Spont.	2.4			Spont.	0.01

of averaged results obtained. A wide range of cytochrome-c contents was observed in these tissues. Most striking, however, is the fact that the order of their cytochrome activities is nearly identical with their relative oxidase activities, the only exception occurring in the case of brain. Even this may be explained by the fact that whole brain was necessarily used in the cytochrome analyses, while essentially cortex was used for oxidase estimation. In experiments with rabbits it can be shown that the gray matter of brain is considerably richer in cytochrome than the white matter.

In addition to the fact that the respiration of tissues is so completely blocked by cyanide (49), these striking relations emphasize the fundamental position of cytochrome-c in cellular respiration.

It has been reported by Holmes (50), using the spectroscope, that cytochrome-c is present only in small amounts in tumor tissue. It has long been known that tumor slices display aerobic glycolysis, *i.e.* they lack ability to oxidize aerobically the lactic acid produced. As early as 1924, Warburg, Posener and Negelein (51) concluded from this fact that in tumors there was a "chronic lack of oxygen". It was of great interest therefore to study the cytochrome oxidase and cytochrome in tumor tissue. Table I also records some average results obtained. It is difficult to state how much of each constituent could be considered sufficient

for normal metabolism. Little can be concluded, therefore, concerning the amount of oxidase found. In the case of cytochrome-c, however, there is little question as to a deficiency, since it is practically absent. With our present knowledge of the importance of the cytochrome system in respiration, it is very possible that this deficiency may be at least one cause of aerobic glycolysis in tumor tissue, and hence be connected with its rapid growth. The view is strengthened by the fact that an embryo, likewise a rapidly growing tissue exhibiting aerobic glycolysis, contains practically no cytochrome-c until shortly before birth.

It is hoped that these methods of analysis will aid in the understanding of other pathological states in which cellular respiratory changes are suspected, and that this paper has emphasized the importance of the cytochrome-cytochrome oxidase system sufficiently to stimulate workers toward the purification and better understanding of its components.

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DISCUSSION

Dr. Stern: I should like to say something about nomenclature, a minor point, but still important at this time when there is so much confusion as to nomenclature.

You called the respiratory ferment of Warburg "oxygen carrying enzyme", whereas the original German thereof by Warburg is "sauerstoffübertragendes Ferment" which correctly translated, would mean "oxygen transferring enzyme" rather than "carrying enzyme".

You said that Warburg has a scheme involving five different iron compounds as catalysts in cell respiration. This particular scheme was, however, set up to explain the exceptional case of the spectrum of *B. pasteurianum*. The general scheme proposed by Warburg incorporates only four iron compounds, *viz.* the respiratory ferment and the three cytochrome components.

You also said that there was no evidence available as to the photochemical behavior of indophenol oxidase, but as far as I remember both Keilin and Dixon have done experiments, though rather crude ones as compared with those of Warburg, in which they showed that the indophenol oxidase was inhibited by carbon monoxide and that the inhibition was reversibly relieved by light of various wavelengths, and I believe it was this evidence which led Keilin and Warburg to assume that these two enzymes are identical.

The last point I want to take up is the question of the occurrence of cytochrome in tumors and its possible relation to the Pasteur phenomenon. Cytochrome, besides being important for cellular respiration, might conceivably be connected with other processes, particularly since the participation of all *three* components in cell respiration seems to be insufficiently established at the present time.

The evidence available at the present time indicates that the Pasteur effect is based on a relation between oxygen tension and glycolysis rather than one between respiration and glycolysis, and that there is a heavy metal-containing link between O_2 and the fermentation system. In agreement with you, v. Euler states that in certain tumors there is almost no cytochrome present. In as much as the Pasteur effect in tumor tissue seems to be disturbed, as evidenced by its aerobic glycolysis, the possibility suggested itself that a cytochrome component might be identical with this Pasteur agent. However, we found that Yale No. 1 tumor, a transplantable malignant tumor, shows a very strong cytochrome-a, -b and c-spectrum. The disappointment was still greater when we examined the behavior of cytochrome towards

ethyl carbylamine. Warburg had shown that this reagent will specifically affect the aerobic glycolysis, raising it to the level of anaerobic glycolysis without changing respiration or anaerobic glycolysis. This is one of the pieces of evidence indicating that the Pasteur agent is an iron compound, because it can be shown that ferrous iron, but not ferric iron, will combine with ethyl carbylamine. However, we have been unable to observe a shift in the cytochrome spectrum when carbylamine was added to heart muscle preparations. Pyridine ferrohemochromogen and myoglobin, on the other hand, react rapidly with carbylamine to yield spectra similar to oxy- or CO-hemoglobin.

Dr. Stotz: Perhaps "oxygen-transferring" enzyme is a better translation than "oxygen-carrying", yet both of these terms are misleading since Warburg believes that only an $Fe^{++} \rightarrow Fe^{+++}$ change is involved in the action of this enzyme.

I would agree that Warburg's concept concerning iron catalysis in respiration involves the three cytochrome components and the "sauerstoffübertragendes" ferment. A possible fifth compound was postulated to explain the existence of the 6390 A band in certain bacteria upon addition of cyanide.

Concerning Keilin's photochemical work on indophenol oxidase, it was stated that Keilin measured the reversible effect of light on carbon monoxide inhibition (partition coefficient), but no attempt was made to construct a photochemical absorption spectrum which was so essential to conclude that indophenol oxidase is identical with Warburg's enzyme.

Dr. Burk: I am a little surprised to hear Stern say that tumor tissue has little of the "Pasteur agent". I should say there is scarcely any tissue that has more; that is, in general, there is no tissue that has a consistently higher Meyerhof oxidation quotient, which means, likewise, a high content of "Pasteur agent".

Dr. Stern: I am afraid we cannot generalize with respect to this point. I should have said that there are undoubtedly some tumors which have a small concentration of Pasteur agent. You remember that Warburg originally thought that aerobic glycolysis in tumors was not due to a particular disturbance of the Pasteur effect, but to what he called a deficient respiration, the respiration being too small in extent to suppress fermentation completely. Later when Murphy and others found spontaneous tumors which showed a higher respiration, which under normal circumstances would have been sufficient to suppress glycolysis, Warburg conceded that the disturbance in tumor cells might be two-fold, namely, due to a deficient respiration and to an inefficient respiration. To which type of the two our tumors belong we don't know, because their metabolism has as yet not been studied. I believe one cannot say that in general all tumors have a high concentration of Pasteur agent. Besides, one might adopt the view that the mere existence of aerobic glycolysis is a symptom indicative of a deficiency in Pasteur agent.

Dr. Burk: I will but repeat that I know of few if any tumors with really low Meyerhof oxidation quotients, even though, as you say, the respiration rate may not itself be definitely low.

Dr. Ball: Though I don't know just what the term "Pasteur agent" refers to, I might add one observation I have been able to make on Jensen rat sarcoma. The spectrograph shows that it contains cytochromes-a,-b, and -c in abundance, and that these cytochromes show the same oxidation-reduction potential relation as those we find in heart muscle.

In the determination of the oxidation-reduction potential of cytochrome-a in heart muscle suspension, I recognize fully the difficulties involved in assigning an accurate value to cytochrome-a, but this observation was made. As the oxidation-reduction potential of the mixture was raised the band of cytochrome-a was found to disappear in an uneven way; that is, there seemed to be a disappearance of the band more in the region 600 to 605 mμ than between 595 and 600 mμ. At that time I felt convinced that cytochrome-a was composed of two components, but the data were not sufficient to make any definite statement in the original article to this effect. A statement was merely made at this time that a band at 595 mμ could not be made to disappear by the addition of further ferricyanide after part of the cytochrome-a spectrum had disappeared. This band may therefore belong to the -a$_3$ component of Keilin and Hartree, in which case it would appear that -a$_3$ has a higher potential than cytochrome-a, as would be expected if its role is that postulated by these workers.

Dr. Stotz: It is interesting that Ball measured the potential of the substance that we still call cytochrome-a, and that the "a$_3$" band had a higher potential, in agreement with Keilin's concept of the function of this substance, namely oxidation of the cytochromes.

Dr. Barker: I should like to point out some discrepancies in the table in which you wish to show a correlation between respiration and cytochrome content. The respiratory rate of heart is not shown, but is certainly far down the list, even though it does contain the largest amount of cytochrome. Furthermore, I notice that brain and skeletal muscle seemed to be in reversed positions.

Dr. Stotz: I hoped this table would point out a rough relation between the cytochrome enzyme system and the respiration of the tissues. When it is considered that the "respiration" of the tissues

as measured *in vitro* varies so greatly according to the methods used, both for the preparation of the tissue and the medium, etc., it is not surprising to find discrepancies in such a comparison as I have made. Certainly the high sensitivity of respiration to cyanide is a more direct way of demonstrating the dependence of respiration on the cytochrome system.

Mr. MacLeod: I think it would be pertinent to assume a more direct relation between oxidase content and aerobic glycolysis. Have you any figures on that point? I think the testis, which is low in the oxidase column, has a relatively high aerobic glycolysis.

Dr. Stotz: It is conceivable that there might be an indirect relation between the concentration of the cytochrome system and aerobic glycolysis, *i.e.* when the system used for the aerobic oxidation of the products of glycolysis is deficient, these products might accumulate. This relation might be inferred from the results with testis, embryo and tumor tissue, but the point has not been studied further.

Dr. Burk: Do you wish to attach any significance to the fact that the columns for cytochrome oxidase and cytochrome content are more or less parallel? I should not think it necessary for both to parallel each other to explain rough parallel respiration rates. Kinetically the oxygen consumption could vary in a regular manner from tissue to tissue depending on either oxidase or cytochrome content. I wonder if you can think of any special significance of these two going together? I would interpret the parallelism to indicate a very close chemical similarity and nearly identical mode of origin, such as exists between chlorophylls-a and -b.

Dr. Stotz: I could not attach any special significance to this fact since, as we have shown in earlier papers, the rate of oxidation of hydroquinone, for example, can be increased by an increase of either constituent. It is possible that they parallel each other because of similar chemical structure.

Dr. Korr: I think that one of the complicating factors in trying to correlate respiratory activity with cytochrome content or oxidase content of tissues represents one aspect of the Warburg-Keilin system which is of great physiological importance and interest. That is, that tissues and cells are capable of abrupt and, in most cases reversible, changes in oxygen consumption, accompanying changes in functional activity (secretion, contraction, etc.) or following the addition of certain pharmacological agents, changes in substrate or other environmental factors. In many cases where these changes have been investigated (in fact, in so many that it may turn out to be a nearly universal principle) they have been correlated with changes in activity of the cytochrome system, as indicated by the magnitude of the cyanide-sensitive fraction of the respiration. The classical example, of course, is the sea urchin egg, the respiration of which, at room temperature, is increased five or six times immediately upon contact with sperm. Before fertilization its respiration is apparently completely cyanide-stable, whereas the added respiration induced by fertilization is nearly quantitatively eliminated by cyanide. The iron systems are present in apparently equal concentrations in the unfertilized and fertilized eggs because respiration in the presence of p-phenylene diamine is the same for both, but become functionally connected or "geared" to the dehydrogenase-substrate systems only on fertilization or activation.

It is of further interest from the point of view of energy transfer that when the increased respiration accompanying functional activity is prevented by HCN the functional activity is also prevented. Even though the respiratory rate be restored to normal by mediators, the potentials of which are close to those of the cytochromes, there is no restoration of functional activity. Whether or not this indicates some specificity of the cytochromes in the mechanism of energy transfer is well worth investigating.

CYTOCHROME OXIDASE

T. R. HOGNESS

The discovery of any enzyme is a question which is always difficult to settle, if no isolation of the enzyme has been effected. Subsequent experimentation has in almost all cases shown that in the earlier work investigators were dealing with complex systems to which much too simple functions and definitions had been applied. While cytochrome oxidase can in some instances be identified with indophenol oxidase, yet the characteristics and distribution of indophenol oxidase, as first described by Vernon (1) and by Batelli and Stern (2), can be ascribed to more than one of the oxidases known at the present time.

Cytochrome oxidase was probably first definitely identified as an enzyme or as a constituent of an enzyme system in 1928 when Warburg and Negelein (3) performed their classical experiments on the photochemically sensitive inhibition of cellular respiration by carbon monoxide, thereby determining the absorption spectrum of the carbon monoxide complex of what Warburg called the *"Das sauerstoffübertragende Ferment."* By showing that oxygen united with this compound, much as it does with hemoglobin, and that the combined oxygen molecule could be displaced by carbon monoxide, he assumed that the oxygen molecule was "activated" by this ferment. With the advent of the Wieland-Thunberg hypothesis of dehydrogenation or hydrogen transfer, the problem of the *"übertragende Ferment"* was overshadowed by the work on the isolation and identification of the dehydrogenases, a field in which Warburg's Institute has played such a leading role.

In 1925 Keilin (4) restudied the myohematins or histohematins of MacMunn, and renamed them cytochromes-a, -b, and -c. He showed that these compounds were widely distributed both in animal and plant cells and that they therefore had an important role in cellular respiration. Keilin demonstrated that the oxidation of cytochromes-a and -c was inhibited by small amounts of KCN, while cytochrome-b was apparently not so affected. Since the addition of this reagent to a solution containing the cytochromes did not change their absorption spectra appreciably, he assumed that the inhibitor did not combine with them directly but with an oxidase through which these respiratory pigments were oxidized by oxygen. The fact that indophenol oxidase was inhibited in a like manner to that of the cytochrome oxidase led Keilin to postulate that indophenol oxidase and cytochrome oxidase were identical substances. Indophenol oxidase was characterized by its ability to bring about the oxidation of *p*-phenylene diamine and of the Nadi reagent by molecular oxygen, but since these reagents are not intermediates in the respiration processes and can be oxidized through the medium of other oxidases (*e. g.* polyphenol oxidase), this test cannot be used uniquely for cytochrome oxidase. Indophenol oxidase has therefore lost its identity. In systems which involve the cytochromes, indophenol oxidase is now replaced by the name cytochrome oxidase, but in some of these systems the arguments applying to the older indophenol oxidase can probably be used for cytochrome oxidase.

Whether cytochrome oxidase and Warburg's original oxygen transporting enzyme are identical is a question which is not yet answered, but the evidence indicates that such is probably the case. There is still a great deal of ambiguity in our conception of both the oxygen transporting enzyme and cytochrome oxidase. For some time it was believed that the cytochromes-a, -b, and -c act in a cooperative way—that one of the cytochromes is oxidized by oxygen by means of the oxidase and the oxidized form of this particular cytochrome in turn oxidizes one of the others which again oxidizes the third. On the basis of the potentials of the cytochromes as determined by Ball (5), this order would be -c, -a, and -b, but Stotz, Sidwell and Hogness (6) showed that the oxidation of cytochrome-c, through the oxidase, apparently takes place independently of cytochrome-a. Although the cytochromes very probably interact with each other, there is the possibility that more than one oxidase will be found, one for cytochrome-c and one or more to cooperate with -a and -b.

Warburg and his co-workers have given the spectral properties of two oxygen transporting enzymes. The one is his original ferment on which he and Negelein carried out their classical experiments in 1928 and found in yeast, particularly the wild yeast *Torula utilis* (3), in the retina of the rat's eye (7), and in *Bacterium Pasteurianum* (8). The carbon monoxide compound of this oxygen transporting enzyme is characterized by two principal absorption maxima at 433 and 592 mμ and several other less defined maxima at 365, 525, and 550 mμ. The other oxygen transporting enzyme is that found by Negelein and Gerischer (9) in *Azotobacter*. The ferro form of this enzyme has an absorption maximum of 632 mμ and the ferri form at 647 mμ. The carbon monoxide compound of the ferro form has an absorption maximum at 637 mμ. The addition of carbon monoxide to the ferro form shifts the position of the maximum 5 mμ toward the red end of the spectrum. Unless otherwise specified we shall denote the oxygen transporting enzyme as that one found originally by Warburg.

Warburg, Negelein and Haas (10), working with *Bacterium Pasteurianum,* found a band at 589 mμ under anaerobic conditions. Upon the addition of carbon monoxide this maximum shifted to 593, a shift of 4 mμ to the red. This value is almost identical with that determined photochemically (592 mμ) on the original oxygen transporting system. Under aerobic conditions this band disappeared and a new one at 639 mμ appeared. These investigators gave evidence that the two bands were not associated with the same substance. No band at 605 mμ (cytochrome-a) was present. In a recently published paper, Keilin and Hartree (11) have succeeded in obtaining one band at about 590 mμ and another at 432 mμ when heart muscle extract was saturated with CO under strictly anaerobic conditions. Under these same conditions, but with no CO, they did not observe a band at 589 mμ as did Warburg, Negelein, and Haas working with *Bacterium Pasteurianum.* On the other hand the latter investigators did not report a band for the carbon monoxide compound at 432 mμ. It is also to be noted that these latter investigators found no band at 605 mμ in the acetic acid bacteria, a band which Keilin and Hartree presume to be due to both cytochrome-a and the oxygen transporting enzyme. Keilin and Hartree assume that the band at 605 mμ originally ascribed to cytochrome-a is a complex one and is due to both cytochrome-a and the oxygen transporting enzyme. No good evidence is advanced for this assumption. The band at 448 mμ they also ascribe to the oxygen transporting enzyme.

Keilin and Hartree working with heart muscle extract, on the one hand, and Warburg, Negelein and Haas working with *Bacterium Pasteurianum* on the other, differ fundamentally in their findings: 1, Keilin and Hartree observed no band at 589 mμ under anaerobic conditions as did Warburg and his co-workers; 2, Warburg found no band at 605 mμ which Keilin claims to be partly due to the oxygen transporting enzyme; and 3, while both found a band at about 590 mμ for the carbon monoxide compound, Warburg, Negelein and Haas did not report any band at 448 mμ which disappeared upon the addition of carbon monoxide. Keilin and Hartree, furthermore, were not able to show that the substance giving rise to the 590 and 432 mμ bands was light sensitive, as it should have been if it were the oxygen transporting enzyme. Nor could they show that reduced cytochrome-c has the ability to reduce the oxidized form of this cell pigment, as it should do if it is cytochrome oxidase.

The ratio of the partial pressure of carbon monoxide to that of oxygen when the carbon monoxide dark inhibition has decreased the activity of the enzyme to 50 per cent varied between 5 and 12 for the oxygen transporting enzyme, while this same ratio for cytochrome oxidase, as determined by the use of the Nadi reagent or paraphenylene diamine, varied from 9 to 24 for a yeast extract and from 3 to 6 for an extract of heart muscle. In neither case was any account taken of a parallel oxidation system which might have taken part in the oxygen uptake.

Polyphenol oxidase, found principally in plants and recently isolated by Kubowitz (12)· and found to be a copper-bearing enzyme, has properties somewhat similar to those of the oxygen transporting ferment, but the fact that its partition constant for CO and O_2 (the ratio referred to above) is uniformly smaller than that for the oxygen transporting system and the fact that this enzyme does not act on the substrates usually present in animal and yeast cells leave cytochrome oxidase as the only other known enzyme which is inhibited by KCN, H_2S, NaN_3 and CO. On this basis Warburg's oxygen transporting enzyme and Keilin's cytochrome oxidase seem to be identical. On one point, however, cytochrome oxidase and the oxygen transporting enzyme differ markedly. Warburg (13) found that the respiration of yeast cells (which was inhibited 40 per cent by 1.5×10^{-4} N HCN) was independent of the oxygen tension between 2.9 and 97 volumes per cent oxygen (total pressure 1 atm.) and from these data he estimated that the equilibrium constant between the ferment and oxygen must have a value greater than 2500. Stotz, Altschul and Hogness (14) measured the dependence of the rate of oxidation of cytochrome by cytochrome oxidase as a function of the oxygen pressure and found that the velocity of oxidation was one-half its maximum value when the oxygen pressure was 40 mm. From these data the equilibrium constant between oxygen and the oxidase can be calculated to be about 20. While it was found that this cytochrome oxidase, prepared from heart muscle, was sensitive to CO, the large discrepancy between the oxygen dissociation constants leaves some doubt regarding the identity of the oxygen transporting enzyme and cytochrome oxidase. The question can only be settled by quantitatively determining the light sensitivity of the carbon monoxide inhibition of what is known to be cytochrome oxidase and comparing this with Warburg's enzyme, or by purifying the cytochrome oxidase to such an extent that the absorption spectrum of the carbon monoxide compound of cytochrome oxidase can be determined directly.

Keilin (15) maintains that cytochrome oxidase is either an insoluble enzyme or is so firmly attached to the cell structure that it is impossible to obtain this substance in solution. Warburg (16) in his Nobel Address said, "In fact, the ferment itself, while still so near is for us as un-

attainable as the material of the stars." Here he had reference to the possibility of obtaining the enzyme in a dissolved form. However, he made this statement in 1931. Yamagutchi et al., (17) on the other hand, have described experiments in which they have been able to obtain an indophenol oxidase in clear solution from yeast, heart muscle, and from the mushroom Lactarius, which retained its activity after repeated filtrations through a Seitz filter. The activities of these extracts were tested for the most part by the strong color reaction with the Nadi reagent, and only a few manometric measurements were reported at a later time by Yamagutchi. No experiments were designed to show that cytochrome-c acted as an intermediary in this oxidation process. These observers found that the catalytic oxidation of the Nadi reagent by their extracts was inhibited by KCN but not by CO. These results, together with many others given by the Japanese school (18), have led them to postulate two intermediary enzyme constituents lying between cytochrome-c and oxygen; one of them acting only as an oxygen transporting enzyme and, like hemoglobin, sensitive to CO; the other sensitive to KCN and acting in a true oxidizing capacity. Keilin repeated the work of the Japanese observers with heart muscle extract and after filtering the extract through a coarse Seitz filter four times obtained a clear solution which had only 11 per cent of the unfiltered extract. From these observations he assumed that with repeated filtration the activity would have disappeared altogether, and therefore concluded that the cytochrome oxidase is insoluble.

The use of the Nadi reagent for determining the activity of cytochrome oxidase is a very ambiguous one and any results obtained by this method cannot be given a unique interpretation, in as much as this reagent can be oxidized by polyphenol oxidase and by traces of metal such as copper. The fact that the oxidase of the Japanese school is inhibited by KCN but not by CO strongly suggests that some metallic impurity such as copper ion is responsible for the effects they have obtained, particularly since no attempt was made to show that this was not the case.

Stotz, Sidwell and Hogness (6), using a heart muscle suspension containing the oxidase, have developed a method for determining the activity of cytochrome-c oxidase more uniquely than has been possible in the past. Besides the Nadi reagent, paraphenylene diamine and hydroquinone may be used as substrates in this reaction. Both the latter substances rapidly reduce oxidized cytochrome-c. Paraphenylene diamine has a lower potential than either cytochromes-b or -c, while the potential of hydroquinone is lower than that for cytochrome-c but above that for cytochrome-b. Hydroquinone should not be able to reduce the oxidized form of cytochrome-b to any appreciable extent. This conclusion was confirmed by a study of the cyanide sensitivity of the reactions involving hydroquinone and paraphenylene diamine. The test involves oxygen, cytochrome-c, and the suspension containing the oxidase, besides the hydroquinone or the paraphenylene diamine. It was shown that with the addition of cytochrome-c to the reaction mixture the rate of oxidation increased until it approached a maximum, evidence that cytochrome-c probably forms a complex with the oxidase. In all tests an excess of cytochrome-c was used. With hydroquinone a very small amount of KCN completely inhibited the catalytic oxidation of the hydroquinone but excess KCN inhibited the oxidation of the paraphenylene diamine only about 85 per cent. 15 per cent of the oxidation of the paraphenylene diamine was ascribed to cytochrome-b, which is auto-oxidizable in the presence of small amounts of cyanide.

Stotz, Altschul and Hogness (14) further showed that carbon monoxide in the ratio of four parts to one of oxygen inhibited the reaction from 55 per cent to 65 per cent in the dark. This inhibition gives a value of about 3 for the partition constant for the oxidase. While Warburg's values for the oxygen transporting enzyme were somewhat larger than this, it must be borne in mind that in the reaction of Stotz, Altschul, and Hogness only that part of the oxidation process in which cytochrome-c oxidase played a role was effective. Oxygen uptake by either cytochrome-b or the yellow enzyme was absent. Making allowance for the other oxidation probably present in Warburg's preparation, the value of the partition constant would be increased to a value which would correspond closely with Warburg's values. The effect of both KCN and CO on the cytochrome-c oxidase is contrary to the findings of the Japanese school and substantiates the view of Keilin that these investigators were dealing with catalytic oxidation of the Nadi reagent by some metal ion.

While the use of hydroquinone in place of other polyphenols or diamines provides a more unique test for cytochrome oxidase, it has the disadvantage that the hydroquinone is not specifically oxidized by reduced cytochrome-c and may also be catalytically oxidized by traces of metal ions and other oxidases. An unambiguous test should involve only reduced cytochrome-c, oxygen and the material to be tested for the oxidase. Using a spectrophotometric method devised by my coworkers and myself at The University of Chicago (19), we have devised such a test. In this case the oxygen uptake is not measured but, instead, the absorption of monochromatic light of 5500 A is used to determine the rate at which the cytochrome is oxidized. Reduced cytochrome has a

sharp absorption peak at this wavelength. In making this test, cytochrome is first reduced by hydrogen in the presence of asbestos coated with palladium as a catalyst. The solution is then filtered and in the absence of the oxidase the cytochrome-c is only very slowly auto-oxidizable. 3 cc. of this solution is placed in an absorption tube and 0.1 cc. of the solution containing the oxidase is then placed in the bottom of the cytochrome solution, by means of a special pipette. The whole solution is quickly shaken and placed in the spectrophotometer, and the rate is determined. Since the absorption coefficients of the oxidized and reduced cytochrome are known, it is not necessary to determine the initial concentration of the reduced cytochrome. If the reaction is too slow it may be expedited with potassium ferricyanide to obtain the final reading which is necessary to calculate the rate. The method does not depend upon a determination of the initial time since the reaction is first order with respect to cytochrome-c.

A typical reaction velocity curve is shown in Fig. 1. The ordinates are the logarithm of the concentration of reduced cytochrome-c and the abscissae, the time. The straight line obtained indicates that the reaction is of first order with respect to cytochrome-c and follows the equation:

$$\frac{-d[Cy(r)]}{dt} = K'[Cy(r)] \qquad (1)$$

or

$$\frac{d \log [Cy(r)]}{dt} = K' \qquad (1')$$

The slope of the curve in Fig. 1 is equal to K'. Since the amount of dissolved oxygen (from air)
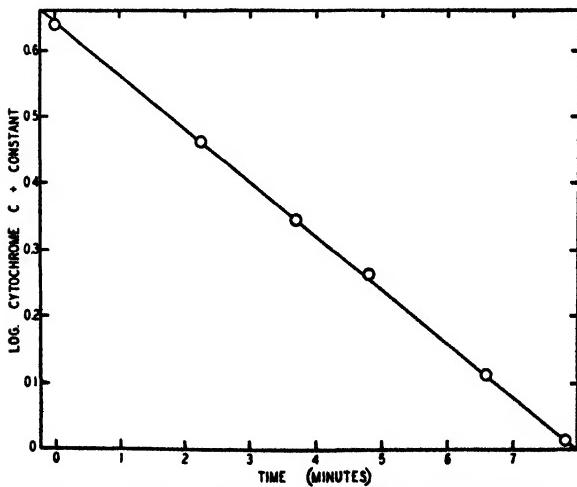
is 17 times the concentration of the cytochrome-c, its concentration may be regarded as remaining essentially constant throughout the course of the reaction. K' in equations 1 and 1' is dependent upon the oxygen and enzyme concentrations. If in all cases the concentration of the oxygen is kept constant one might expect that K' is equal to $K(E)$. (E) here represents the enzyme concentration. If this were true, then, plotting K' against relative enzyme concentrations should give a straight line. This is shown to be the case in Fig. 2.
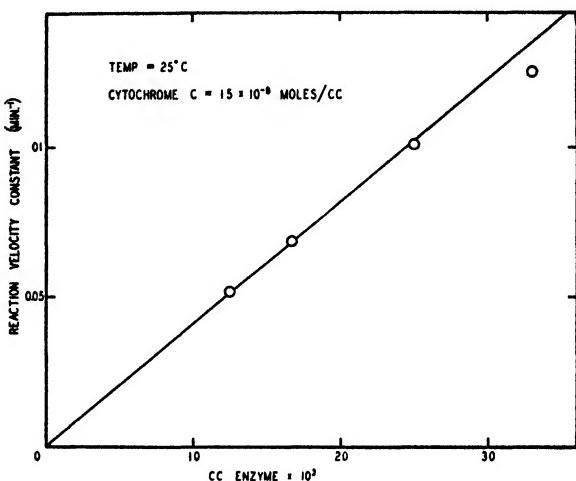
Fig. 2. Rate of oxidation of reduced cytochrome-c as a function of the oxidase concentration.

In the rates as plotted in Fig. 2 corrections were made for the auto-oxidation. The straight line obtained corroborates the assumption that the reaction is first order with respect to both the cytochrome and the oxidase in low concentrations:

$$\frac{-d[Cy(r)]}{dt} = K[Cy(r)](E) \qquad (2)$$

K in this equation is still dependent on the oxygen concentration, which was kept essentially constant in all our experiments.

This test for cytochrome, while much more specific than the manometric method which involves hydroquinone, suffers some disadvantages. Only one determination can be made at a time. With two men working each test requires approximately one-half hour. The amount of the oxidase necessary for the test is much less than that necessary for a manometric determination. With such small concentrations of enzyme, inhibitors present as impurities can become troublesome. As yet, no difficulties have been encountered on this score. However, the small concentration

Fig. 1. Rate of oxidation of reduced cytochrome-c.

of enzyme necessary has the advantage that small scale separations may be used to advantage.

Using the spectrophotometric method for testing the activity of the enzyme my collaborators, Altschul and Abrams, and I have succeeded in obtaining from yeast a soluble preparation which has a high oxidase activity. The data shown in Fig. 1 and 2 were made on this soluble preparation.

Not all yeasts can be used to give this preparation and elaborate tests on many yeasts were necessary before a suitable sample was found. Nor is the oxidase content from any one source constant, so tests must consequently be made on every fresh sample obtained. Both bakers' and brewers' yeast have been found to contain this enzyme. The enzyme is very labile and the conditions for its preservation or destruction have not yet been ascertained. In the wet state the yeast retains its activity over a period of at least one month when kept in a refrigerator. In the dry state yeast loses its activity quite rapidly, one-half the activity disappearing in a week's time.

The active preparation is made by first drying the yeast overnight and as soon after drying as possible making a *"Lebedew-saft"* of one part of the dried yeast to three parts of water by weight. The *"Lebedew-saft"* is allowed to digest for five hours, and is then centrifuged for a half hour in a high speed centrifuge at about 10,000 times gravity. It was found that this centrifugal force was almost as effective in removing suspended material as was 35,000 G obtained with a Beam's ultracentrifuge. Higher centrifugal force would throw the higher molecular weight proteins out of solution.

After centrifugation the whole of the oxidase activity remains in solution. The enzyme may then be concentrated by fractional precipitation with ammonium sulfate, and subsequently dissolved again in buffer or distilled water.

The conditions for the precipitation depend upon the kind of yeast used and have not been fully worked out by us yet. In one instance an active preparation was made which had undergone two ammonium sulfate and two alcohol precipitations.

These properties of the enzyme make it appear that it is a protein with which we are working, and that our results are not due to catalysis by inorganic material. We have been much concerned over the possibility that the catalytic action of our yeast extract was due to some metal ion, particularly in view of Keilin's statement that metal ions such as those of iron and copper catalyze the oxidation of reduced cytochrome-c. We have carried out a number of experiments to clarify this point and have come to the conclusion that in our ex-

TABLE I

Exp.	Catalyst	Amount of Catalyst	K (Min^{-1}) $\times 10^3$	$(K - K_1) \times 10^3$	$(K - K_7) \times 10^3$
1.	—	—	10	—	
2.	Fe^{++}	1.5×10^{-10} mole	9	−1	
3.	Fe^{++}	1.5×10^{-10} mole	11	+1	
4.	Fe^{++}	1.5×10^{-9} mole	11	1	
5.	Cu^{++}	1.8×10^{-8} mole	14	4	
6.	Mn^{++}	1.7×10^{-8} mole	14	4	
7.	Inactive Yeast Extract	0.005 cc.	18		—
8.	Fe^{++} + Yeast Extract	1.5×10^{-9} mole 0.005 cc.	20		2
9.	Cu^{++} + Yeast Extract	1.5×10^{-8} mole 0.005 cc.	21		3
10.	Mn^{++} + Yeast Extract	1.5×10^{-8} mole 0.005 cc.	16		−2
11.	Active Enzyme Solution	0.0024 cc.	113	103	

Total Cytochrome = 4.5×10^{-8} mole   Total Volume = 3.1 cc.

periments metal ion catalysis is not responsible for the oxidation of reduced cytochrome-c.

The catalytic actions of ferrous, cupric, and manganous ion on the oxidation of reduced cytochrome-c were determined and, contrary to Keilin's contentions, cytochrome-c is not catalytically oxidized by these ions. The results of these tests are given in Table I. $K - K_1$ in the fifth column of the table is the rate with the metal ion minus the rate of auto-oxidation, and represents the catalytic activity of the metal ion in question. In some cases the concentration of the metal ion was as much as one-tenth that of the cytochrome-c itself. The small positive effects are negligible. Keilin used the Nadi reagent, paraphenylene diamine, or hydroquinone is his tests, and iron and copper ions catalyze the oxidation of these substrates but not, as Keilin assumed, with the cooperation of cytochrome-c. These same ions were then added to a yeast extract which was obtained in the same way as was the active preparation, but from an inactive yeast sample. These mixtures also showed no catalytic action. This shows that a combination of any one of these ions with some protein could not be responsible for the oxidation. The activities of these solutions are given in the table. $K - K_7$ is the rate of oxidation of reduced cytochrome-c by the metal ion and protein minus the oxidation by the protein alone. In both cases, with and without protein, the ions of iron, copper and manganese are without effect, even in concentrations as high as one-tenth that of the cytochrome-c.

An active sample of the oxidase was allowed to dialyze for 48 hours, after which it retained 75 per cent of its activity. The loss could be accounted for on the assumption that it was due to the action of proteolytic enzymes in the unpurified sample.

The catalytic activity of the preparation was destroyed after heating for 10 minutes at 55° C.

In one case a sample of yeast was allowed to digest for three hours and showed no activity. However, with a 5 hour digestion high activity was obtained. These latter facts support the view that a real enzyme is acting as the catalytic agent in the oxidation of reduced cytochrome-c.

The reasons for believing the extract of yeast to contain true soluble cytochrome-c oxidase may be summarized as follows:

(1) The solution does not lose its activity when subjected to a centrifugal force of 35,000 G for one hour. After this treatment the solution appears to be perfectly clear.

(2) The activity can be removed together with the proteins precipitated with ammonium sulfate. The active part of the precipitate can be redissolved in buffer solution or in distilled water.

(3) The ions of iron, copper, and manganese do not catalyze the oxidation of reduced cytochrome-c by oxygen, either by themselves or when mixed with a protein solution obtained from yeast. The catalysis could not be due to these ions. This becomes more apparent when one realizes that as little as 1/500 cc. of active yeast extract is added to 3 cc. of cytochrome solution to be tested.

(4) The activity of the solution is completely removed by heating the extract for 10 minutes at 55° C.

(5) The activity is not removed by dialysis.

(6) The obtaining of an active extract is dependent upon the autolysis of the yeast. If the yeast cells are not broken after digestion there is no activity.

As might be expected the addition of KCN has a strong inhibiting effect on the oxidase. The extent of this inhibition is shown in Fig. 3. The slope of any given line is to be compared with the slope of line 6 in order to appreciate the cyanide inhibition. As small a concentration as 3.3×10^{-8} mole per cc. completely inhibits the activity of the oxidase. A concentration of KCN of 1.6×10^{-8} mole per cc. inhibits the reaction 40 per cent and 90 per cent inhibition is affected with a concentration of 2.3×10^{-8} mole per cc. (The effect of heat treatment is also shown graphically in this same figure. In this case the slope

Fig. 3. Effect of KCN and heat on the activity of cytochrome-c. Ordinate: log. cytochrome-c + constant. Abscissa: time (minutes).
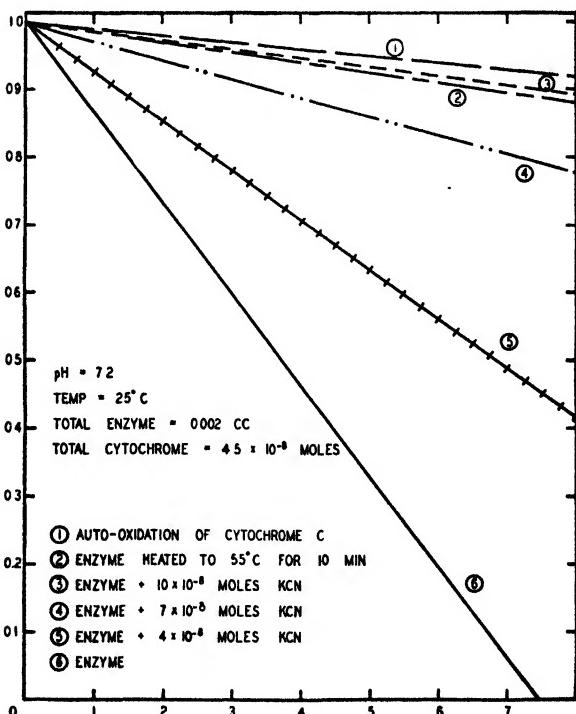
of line 2 is to be compared with that of line 6.) It may be of interest to note that the concentration of the cyanide necessary for inhibition is of the same order of magnitude as that of the cytochrome-c. This would suggest the possibility of the KCN combining directly with the reduced cytochrome-c if it were not for the fact that Keilin found no change in the position of the absorption band of reduced cytochrome-c upon the addition of cyanide. However, he probably would not have been able to observe any small shift in the band with the small dispersion spectroscope he used.

The activity of the oxidase was completely inhibited by CO in the proportion of 87 parts CO to 13 parts O_2 by volume. In these experiments 1 cc. of reduced cytochrome-c and 1 cc. of the oxidase were saturated anaerobically with pure CO. To this saturated solution 4 cc. of a buffer solution was added which had been previously saturated with a mixture of 4 parts CO to 1 part O_2. The rate of oxidation was only as great as the auto-oxidation of the cytochrome. These experiments were repeated using H instead of CO and the oxidase displayed its usual high activity.

In keeping with a general program we have laid out we have measured the heat of activation for the oxidation of reduced cytochrome-c by oxygen, with the oxidase, and have found a value of about 12,000 calories. This value was obtained on a crude enzyme extract using a temperature range of only 15°, and is therefore only tentative.

Cytochrome oxidase is of particular importance to the chemist because of its position at the end of the respiratory or the hydrogen transporting chain. It is that enzyme which activates the oxygen. Through this enzyme the chemist may learn something about the mechanism of oxidation of substances containing metal ions by oxygen, a process about which he is almost wholly ignorant.

The oxygen molecule must undergo a change in valence number of four, while cytochrome-c undergoes a valence change of only one. It may be that four steps are involved before oxygen can be converted to water. Formally the different stages of reduction of oxygen may be represented by the compounds HO_2, H_2O_2, H_3O_2 (H_2O + OH), and H_2O or their derivatives. We have shown that cytochrome enters into the reaction as a first-order constituent. This means that the first product formed would be HO_2 or its derivative, if the oxidation of the oxidase does not take place simultaneously with that of the cytochrome-c. However, since the oxidase is very probably also an iron-bearing compound, it may be oxidized simultaneously from the ferrous to the ferric state and the first intermediate would then be H_2O_2. There is no evidence that H_2O_2 is formed by the cytochrome system, but it must be

borne in mind that this system has never been freed from catalase. Until the components of this system have been isolated we shall never be able to study this problem in an unambiguous manner.

The problem of the function of the cytochromes and cytochrome oxidase is still far from being solved. Progress has been hindered by inability to obtain the oxidase in solution. We now believe that this first step has been accomplished. We may hope that the isolation of the enzyme, which is now under way, will not require too long a time.

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DISCUSSION

Dr. Müller: Could you tell us some more about the difficulties and how you overcame them in preparing this enzyme recently?

Dr. Hogness: That is something which is still not understood. There does not seem to be any apparent reason for our failure to obtain the enzyme consistently. We thought at first that there was something in our technique responsible for it, and we carefully examined all of our reagents, purified them, and used different samples of distilled water and tap water, but still were not able to find the answer.

There are probably two factors involved. The

yeast must autolyze, but all the time this process is going on, the proteolytic enzymes are also working. There is a race between these two processes and the principal difficulty appears to be that of getting the conditions optimum for rapid autolysis and slow hydrolysis of the enzyme.

I am not absolutely sure of all of this, since I base my conclusion on one or two preliminary experiments. Formerly we made a five hour extraction and got nothing, but found that after fifteen or twenty hours an active extract was obtained. Longer time than this gave less active preparations.

In a sample which was not active, the yeast cells appeared to be whole. With an active sample, the yeast cells appeared to be very much shrivelled up and broken. I think that sufficient autolysis is the secret of the whole thing. I cannot give the conditions for it now, since the process has not yet been standardized for one particular yeast sample.

Dr. Abramson: In the early part of your paper you mentioned that Keilin filtered the Japanese extract four times and found the activity reduced to 11 per cent. On the basis of the reduction of activity after filtration he concluded the compound was insoluble. I do not quite follow that.

Dr. Hogness: I quite agree with you. If, for example, he had plotted activity against filtration and shown that a continuous filtration would have extrapolated to zero activity he would have had a better argument. He made one filtration which reduced the activity to thirty or forty per cent, and then made three more filtrations.

Dr. Abramson: In very highly active biological materials which have high molecular weights you would very rarely get no adsorption with soluble compounds. Do you think the Japanese might have had the substance in question?

Dr. Hogness: They might have. The difficulty there is to prove that the Nadi reagent was not oxidized through the medium of metallic ions. They made a sodium chloride extraction and presumably, if this sodium chloride were not pure, it might introduce iron or copper. They might have had a soluble oxidase. I have no way of judging, except that we were never able to get a soluble oxidase from heart muscle as the Japanese workers did.

Dr. Stern: We have worked exclusively with heart muscle; there we find that the cytochrome oxidase activity of heart muscle appears to be intimately bound up with macromolecular material.

The main confusion in the discussion about solubility and insolubility of oxidases appears to be due to the fact that the statement that cytochrome oxidase as an insoluble enzyme was made at a time when the macromolecular materials of the type of plant or animal viruses had not yet been discovered. In other words, at that time it was assumed that if a material is retained by a filter candle, there is definite proof that the material is not present in solution; whereas if you take any number of virus proteins and filter them through candles or graded membranes the filtrate will be inactive although it is now generally accepted that the particles are present in molecular dispersion. The cytochrome oxidase of heart muscle seems to belong to that class. Batelli and L. Stern differentiated between what they call the main respiration of tissues and the "accessory" respiration; their claim was based on the observation that in tissue extracts there is found a certain fraction of the total respiration. Warburg later reinvestigated and came to the conclusion that this "accessory" or extract respiration only amounted to such a small fraction of the main tissue respiration that although it might exist, it is quantitatively insignificant. In order to assess the significance of Hogness' new enzyme it would be necessary to find out how large the contribution of his soluble enzyme is to the total yeast respiration and also, perhaps, to examine the possibility that the phenomena which he has observed might not perhaps be due to the fact that a small amount of the macromolecular material has escaped his sedimentation or other purification procedures.

Dr. Hogness: With heart muscle extract we are not able to get any activity in solution after centrifugation. In the case of yeast extract, however, all the activity is retained. We therefore discarded heart muscle as a source of material and went over to the yeast for that purpose. What we mean by solubility is relatively immaterial for our problem. Our problem is to obtain the enzyme in a form which we can fractionate by precipitation and I think we have it in that form. That is all I mean by solubility. But it may be, of course, that there are two different cytochrome oxidases and this is not the cytochrome oxidase which is functioning in heart muscle.

Dr. Ball: Can you give us any values as to the relative activity of yeast enzyme and heart muscle preparations, say per gram of protein?

Dr. Hogness: I am sorry that I cannot give you the figures. This work has been done only very recently and we have not got around to that yet. The activity is such that we have found that the amount of impure protein necessary for a single test amounts to about 6 micrograms, which means that the enzyme must have a very high activity. I cannot give the relative values for heart muscle.

Dr. Ball: Did you make any spectroscopic observations on concentrated enzyme solutions?

Dr. Hogness: No. We have not had enough of the material to get a highly concentrated solution. We have worked with very small quantities, only a few cc. being necessary. Since we need

only 1/500th of a cc. to make the test you see how little is required.

Dr. Ball: What was the source of your cytochrome-c? I ask that because there appears to be some evidence, though admittedly not very good, that the cytochrome-c in yeast and in heart muscle may be different.

Dr. Hogness: In preparing cytochrome-c we used Keilin's directions just as he describes them.

Dr. Barker: When you were studying the effect of the metals on the possible stimulation of the oxidase activity, if I interpreted the table correctly, you added an excess of certain heavy metals to your solution. Supposing the oxidase actually has a metal in it which is an integral part of the molecule it is functioning with, would the addition of extra metal affect the activation?

Dr. Hogness: Actually we have never tried the effect of metal on the active solution. What we were trying to do was to overcome any criticism that the activity was due to metal ions and not to an oxidase.

Dr. Barker: Do you have any evidence as to whether copper is or is not involved in the activity of the oxidase?

Dr. Hogness: No.

Dr. Stern: I believe the discrepancy between your results and those of Keilin with respect to copper may perhaps be explained by the fact that he used much larger concentrations of copper than you did.

Dr. Hogness: We could not very well use a larger concentration. We had already employed concentrations as high as 10 per cent of the concentration of cytochrome-c. If we were to add ferric iron in large concentrations we would obtain a direct oxidation, and not catalysis. If we now compare the values of the concentration of iron used by Titoff for the oxidation of sulphite with those used by us we find that ours is hundreds of times greater. Keilin used a manometric method, probably with paraphenylene diamine or hydroquinone as substrates. These substances are oxidized through iron but not through the medium of cytochrome-c. He assumed that it was the cytochrome-c which was oxidized by the metal and that the cytochrome-c oxidized the hydroquinone, whereas the metal probably oxidized the hydroquinone directly.

Dr. Stern: So far as I know, the experiments of Keilin to which I have referred were done spectroscopically by adding a considerable amount of copper sulphate to solutions of reduced cytochrome-c.

Dr. Hogness: I can hardly understand how that would take place for the reason that the potential for the cuprous-cupric electrode is considerably lower than that for cytochrome-c. I can understand it with cytochrome-b but not with cytochrome-c.

I am quite sure we never had any such concentrations in our enzyme, and that is the point which I wish to emphasize.

REACTIONS IN WHICH CYTOCHROME AND YELLOW FERMENTS TAKE PART

Frank Urban

Keilin (1) found that cytochrome became reduced in the wing muscles of living insects when these muscles were active. Urban and Peugnet (2) showed that cytochrome-c and yellow ferment transferred electrons during individual twitches of a (frog) muscle. Millikan (3) observed that the oxygen depot of the muscle (oxymyoglobin) discharged oxygen during tetanic activity of a (cat) soleus muscle. He concluded that "muscle haemoglobin acts as a short time oxygen store to tide the muscle over from one contraction to the next".

This evidence led Urban to formulate the following hypothesis[1a] of muscular contraction:

$$\text{glyceric aldehyde phosphate} \xrightarrow{\text{carriers}} \text{myosin} \xrightarrow{\text{carriers}}$$

cytochrome-c oxidase ⟶ oxygen

⟶ cytochrome $\xrightarrow{\text{carriers}}$ pyruvic acid
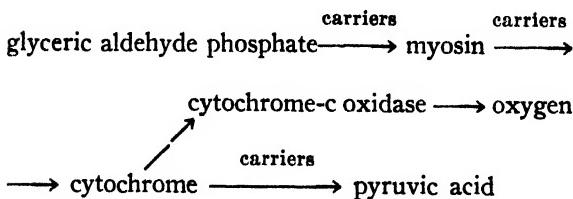
the arrows indicating the flow of electrons (or, hydrogen *atoms*).[1]

In this concept of muscular contraction pyruvic acid, the oxidation product of glyceric aldehyde phosphate (Warburg (4), Meyerhof (5)), assumes the role of a "substitute" for oxygen. That is, the electrons which normally flow to oxygen will flow to pyruvic acid, whenever oxygen is scarce or absent, thereby forming lactic acid. The flow of electrons to myosin constitutes the contraction reaction; the flow of electrons away from myosin constitutes the relaxation reaction. The scheme purposely makes no allowance for the "trigger" mechanism which initiates the contraction reactions.

Some support for this hypothesis has been obtained in experiments carried out in the absence of cytochrome, *i.e.* in studies of the contraction reaction glyceric aldehyde phosphate → myosin alone (expts. B-54, 1-2-3-4-5-6). It is hoped to present this work at some future time. The present paper deals with the electron "outlet" part of the hypothesis, namely with studies of reactions involving cytochrome, and lactic and pyruvic acids.

The cytochrome-c oxidase preparation of Keilin and Hartree (6) is ideally suited for the study of the lactic and pyruvic acid reactions. It is prepared from fresh pig hearts. As Keilin and Hartree have pointed out, the preparation contains numerous catalytic proteins, in addition to cytochrome and cytochrome-c oxidase. Indeed, it is perhaps permissible to look upon this mosaic of catalysts as a co-ordinating unit of physiological reactions.

I have introduced some minor changes in Keilin and Hartree's method of making the cytochrome-c oxidase preparation. Because of the high spring and summer temperatures prevailing in St. Louis, the entire preparation was carried out in a cold room at + 1° C.; the grinding, however, was done at —2° C. The fresh pig hearts were packed in ice at the slaughter house and immediately taken to the cold room. The preparation was started immediately. All the materials and equipment needed were at +1° C. This included the mechanical grinder, centrifuge, mortar, centrifuge cups and bottles, sand, solutions, beakers, pipets, balances, cylinders, mechanical stirrer, etc.

Two cleaned and ground hearts were washed ten times with distilled water (mechanical stirrer), ground in a mortar for 45 min. (by hand) with the solutions prescribed by Keilin and Hartree (600 cc. of M/25 phosphate in two portions). This was followed by centrifugation (2 min. at 1440 r.p.m.) The cytochrome-c oxidase preparation was precipitated from the supernatant fluid by the addition of 100 cc. of M/5 acetate buffer of pH 5.0, followed by centrifugation for 15 min. at 1800 r.p.m. The pH of the supernatant fluid was then about 5.8. Ten washings (by stirring with a glass rod) and centrifugations followed, 50 cc. of M/15 phosphate buffer of pH 5.8 being used each time. The supernatant liquid was sucked off (water suction pump) as completely as possible after each centrifugation. Finally, after these ten washings, the oxidase preparation was suspended in 35 cc. of M/10 phosphate buffer of pH 7.3.

Thus the preparation had undergone a total of 21 washings, 10 before and 11 after the grinding operation in the mortar.

Lactic ferment.

Thus prepared, the cytochrome-c oxidase preparation shows zero oxygen consumption. This is very desirable whenever reactions involving a small oxygen consumption are to be studied. I shall refer to it as the "fresh preparation".

[1a] Weber states in Naturwissenschaften *27*, 38 (1939): "The active contraction of living muscle appears to be due to a chemical reaction of myosin with unknown substances originating in the functional metabolism of the muscle".

[1] The hydrogen *atom* must not be confused with the hydrogen *ion*. By the loss of one electron, the hydrogen *atom* becomes a hydrogen *ion*. Glyceric aldehyde phosphate is in reversible equilibrium with hexose diphosphate (Meyerhof (5)). Hexose diphosphate is ultimately derived from glycogen, either directly or indirectly.

After about 2 days at $+1°$ C., the preparation, which at first consumes no oxygen unless substrate is added, has acquired an oxygen consumption of about 10 mm³. per 3 hrs. per 1 cc. of preparation which proceeds at constant rate in the Warburg apparatus at 37° C. for the duration of the measurements (3 hrs.). Such a preparation will be referred to as an "old preparation". Experiments performed with old preparations will be specially designated.

If the cytochrome-c oxidase preparation contains an enzyme which can catalyse the reaction

$$2H + \text{pyruvic acid} \rightleftharpoons \text{lactic acid}$$

one should expect the addition of lactate to the fresh preparation to result in the production of pyruvic acid and the liberation of 2 hydrogen atoms. The latter can combine with (reduce) a prosthetic group only, since all diffusible co-ferments have been removed from the preparation by 21 washings. The prosthetic group, in turn, might transfer the two hydrogen atoms to oxygen. If, however, carriers were added, the prosthetic group might in turn transfer the hydrogen atoms to the carriers. The reaction would continue to run to the left as long as both reaction products, *i.e.* hydrogen atoms and pyruvate, were removed. If only one of the reaction products were removed, the reaction would come to a standstill (equilibrium). I shall call this enzyme "lactic ferment".

(Expt. 176,1).

1 cc. of fresh cytochrome-c oxidase preparation in M/10 sodium phosphate buffer of pH 7.3 + 0.3 cc. H_2O + 0.2 cc. lactate (= 1.08 mg. lactic acid) {inset 0.2 cc. of 20 p.c. NaOH to absorb CO_2} showed an oxygen consumption of 34.3 mm.³ in 139 min. Gas phase: pure oxygen. Temp. 37° C. Control: the same components, but without mixing, showed zero oxygen consumption.

At the end of 139 min., 50γ of lactic acid had disappeared (analytically determined).

For complete combustion to CO_2 and H_2O, the calculated O_2 consumption is 37.5 mm³. (found: 34.3 mm³.).

Since the reaction showed no signs of coming to completion at the end of 139 min., it appears that hydrogen atoms liberated by lactic acid are combining with oxygen to form water, while pyruvic acid is being oxidized to CO_2 and H_2O. The reaction is quite slow, suggesting that the equilibrium ratio pyruvic/lactic is very small. The experiment also indicates that there is present, in addition to lactic ferment, an enzyme which oxidizes pyruvic acid to CO_2 and H_2O. I shall speak of the enzyme as "pyruvic ferment".

(Expt. 147, 1).

A small three-legged vessel contains 1 cc. of fresh cytochrome-c oxidase preparation in the first leg, 0.1 cc. of sodium lactate (about 5 mg., prepared from crystalline zinc lactate) in the second leg, and nothing in the third leg. The vessel is filled with oxygen-free hydrogen gas and sealed.[2] The contents are mixed by tilting. There is no evidence of any reaction leading to the appearance of the *a*α, *b*α and *c*α cytochrome bands.[3] It will appear below that repetition of this experiment, but with the addition of suitable carriers, leads to the prompt appearance of the *a*α and *c*α bands of reduced cytochrome.

The spectroscopic experiment 147, 1 can be reconciled with the respiration experiment 176, 1 by the interpretation that both lactic and pyruvic ferments are oxytropic ferments, *i.e.* that they may react with oxygen directly.

One is reminded of the yellow ferments of Warburg (8), some of which react with oxygen, as well as with carriers. It is thought that additional spectroscopic evidence, supplemented by isolation of the individual ferments, will definitely establish the chemical nature of lactic and pyruvic ferment.

Pyruvic ferment.

The above experiments have indicated that pyruvic ferment oxidizes pyruvic acid to CO_2 and H_2O. This will now be demonstrated with pyruvic acid as substrate.

(Expt. 181, 11-12).

1.5 cc. of fresh cytochrome-c oxidase preparation + 0.3 cc. pyruvate (2.3 mg.) in the side arm of the Warburg vessel + 0.2 cc. of 5N H_2SO_4 in the other side arm + 0.2 cc. of 20 p.c. NaOH in the inset. Gas phase: pure oxygen. Temp. 37° C. Mixing of pyruvate and oxidase after temperature equilibrium.

The oxygen consumption was found to be 17.9 mm³. in 90 min. (average of two determinations).

A control, in which cytochrome-c oxidase and pyruvate were not mixed showed zero oxygen consumption.

A preliminary determination of the R.Q. has been made and the value 1.2 has been found (average of two determinations). The theoretical R.Q. is 1.20.

[2] The result is the same if oxygen-free nitrogen is used. The oxygen-free hydrogen was prepared by passing hydrogen gas over platinized asbestos at dull red heat.
[3] I have followed the new nomenclature of Keilin and Hartree (7).

Activation of pyruvic and lactic ferments by vitamin C.

The above experiment (181, 11-12) is repeated, but with the addition of 20 γ of vitamin C to the pyruvate in the side arm of the Warburg vessel. After temperature equilibrium has been established, the cytochrome-c oxidase preparation will be mixed with pyruvate + 20 γ of vitamin C.

(Expt. 180, 3-5).

The effect is surprising. The presence of 20 γ of vitamin C has increased the oxygen consumption in 90 min. to 78.8 mm^3. (17.9 mm^3. without vitamin C). After deducting a blank of 14.8 mm^3. for vitamin C alone, in the absence of pyruvate (Expts. 182, 2; 183, 7) there remains an oxygen consumption of 63.2 mm^3., a more than threefold increase.

The large blank correction of vitamin C is also surprising. The calculated O_2 consumption for the conversion of 20 γ of ascorbic acid (vitamin C) to dehydroascorbic acid is about 1.3 mm^3. Instead 14.8 mm^3. were consumed. This suggests that proteins are being oxidized. The question must be left open for the present.

The large (threefold) O_2 consumption in the presence of pyruvate points to the activation of pyruvic ferment by vitamin C. This viewpoint is confirmed by Expts. 179, 10-12 which were carried out exactly like the previously described experiments 180, 3-5, except that an old cytochrome-c oxidase preparation was substituted for the fresh preparation. The old preparation had been kept in the cold room for 4 days.

(Expt. 179, 10-12).

1.0 cc. of old cytochrome-c oxidase preparation + 0.2 cc. pyruvate (= 2.3 mg.) and 0.1 cc. of vitamin C (20 γ) in the side arm of the Warburg vessel + 0.2 cc. of 5N H_2SO_4 in the other side arm + 0.2 cc. of 20 p.c. NaOH in the inset. Gas phase: pure oxygen. Temp. 37° C.

Pyruvate + vitamin C were mixed with cytochrome-c oxidase preparation, after temperature equilibrium had been established. The oxygen consumption in 75 min., after applying a correction of 14.8 mm^3. for the vitamin C blank, was 26.9 mm^3. The oxygen consumption in a control experiment without vitamin C was 26.8 mm^3.

The data show that the activating effect of vitamin C has disappeared.

The same (old) preparation, however, responded to 20 γ of vitamin C when *lactate* was used as a substrate.

(Expts. 178, 3; 179, 8).

1.0 cc. of old cytochrome-c oxidase preparation + 0.2 cc. lactate (1 mg.) and 0.1 cc. vitamin C (20 γ) in the side arm of the Warburg vessel +

0.2 cc. of 5N H_2SO_4 in the other side arm + 0.2 cc. of 20 p.c. NaOH in the inset. Gas phase: pure oxygen. Temp. 37° C.

Lactate + vitamin C were mixed with cytochrome-c oxidase preparation, after temperature equilibrium had been attained. The oxygen consumption was 37.8 mm^3. in 75 min. (corrected for the vitamin C blank by deducting 14.8 mm^3.). The oxygen consumption in the absence of vitamin C was 9.8 mm^3. in 75 min. This represents a fourfold increase in the presence of vitamin C.

Since pyruvic ferment was inactive in the same (old) cytochrome-c oxidase preparation, it seems permissible to speak also of an activation of lactic ferment by vitamin C.

Oxidation of pyruvic acid by yellow ferment 9.

When fresh cytochrome-c oxidase and pyruvate are mixed anaerobically, no change in the spectrum occurs (Expt. 147, I). If the experiment is repeated, this time with the addition of 20 γ of oxidized lactoflavin to 1 cc. of cytochrome-c oxidase preparation, the $a\alpha$ band appears soon and the $c\alpha$ band in 5 min. (Expt. 170, I). Obviously the outlet to oxygen of this reaction is by way of cytochrome. The experiments may be interpreted in one of two ways. Either lactoflavin acts as a carrier between pyruvic ferment and cytochrome; or, lactoflavin acts both as a carrier and the co-ferment of a catalytic protein present in the cytochrome-c oxidase preparation, the co-ferment + catalytic protein constituting a new enzyme capable of oxidizing pyruvate. I shall now adduce evidence to show that we are probably dealing with a new enzyme to which I shall refer as yellow ferment 9.[4]

(Expt. 175, 11-12).

1 cc. of fresh cytochrome-c oxidase preparation + 0.2 cc of lactoflavin (= 50 γ) in one side arm of the Warburg vessel + 0.2 cc. of pryuvate (= 2.73 mg.) in the other side arm + 0.2 cc. of 20 p.c. NaOH in the inset. Gas phase: pure oxygen. Temp. 37° C. Measurements were made in the darkroom.

After temperature equilibrium had been established, lactoflavin, pyruvate and the preparation were mixed. The O_2 consumption in 85 min. was 30.6 mm^3. (average of 2 determinations).

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[4] Warburg (8) has listed 5 yellow ferments. To this number there should be added

    6. The yellow ferment of R. Kuhn and H. Rudy (Ber. chem. Ges. *69*, 2557)

    7. The flavoprotein of H. S. Corran and D. E. Green (Biochem. J. *32*, 2231 (1938)).

    8. The flavoprotein of H. S. Corran, D. E. Green and F. B. Straub (Biochem. J. *33*, 793 (1939)

   10. Fumarate hydrase of F. G. Fischer, A. Roedig and K. Rauch. Naturwissenschaften *27*, 197 (1939).

Control: an identical set-up in which the components were not mixed, showed zero O_2 consumption in 85 min. Analytical determinations:

pyruvic acid oxidized   0.41 mg.
lactic acid formed   0.09 mg.

From these analyses one calculates an oxygen consumption of about 1/3 O_2 per molecule of pyruvate oxidized.[5] The oxidation product is unknown.

It is noteworthy that we are not dealing here with a "dismutation" of pyruvic acid, since the quantities of pyruvate oxidized and of lactate formed are not equivalent. A "dismutation" of pyruvic acid in the presence of lactoflavin has been observed by Lipmann in *Bact. Delbrückii* (9).

Another curious feature of Expts. 175, 11-12 is this: the preparation behaves as if lactoflavin had inactivated pyruvic ferment.

The effect of vitamin C on yellow ferment 9 has been investigated, but no definite conclusion has been reached as yet. Vitamin C appears to have an inhibitory effect, if any. (Expts. 173, 1/6).

I have also observed in anaerobic spectroscopic experiments that succinate has no effect on the rate of the appearance of the *ca* band of cytochrome (Expt. 172, I, II), in the presence of lactoflavin and pyruvate.

Further work is necessary to clear up these points.[6]

The oxidation of pyruvic acid by aneurine ferment.

Lipmann (9) discovered the oxidation of pyruvic to acetic acid, B_1-pyrophosphate acting as co-ferment of aneurine ferment. He believed (9) that "between aneurine ferment and oxygen an unknown carrier is interposed".

I have found that (Expt. 166, I) B_1-pyrophosphate reacts promptly with cytochrome. When 1 cc. of fresh cytochrome-c oxidase preparation and 25 γ of B_1-pyrophosphate are mixed anaerobically in the presence of 2.5 mg. of pyruvate, the *ca* band of cytochrome appears after 5 min. Mg^{++} in a final concentration of 2 mg. p.c. slows the reaction, *ca* appearing after 6 min. 18 sec. (Expts. 168, V, VI). The aneurine ferment of Lipmann therefore seems to be present in the cytochrome-c oxidase preparation.

[5] The oxidation of pyruvate to acetate requires 1/2 molecule of O_2; the oxidation of pyruvate to CO_2 and H_2O requires 2½ molecules of O_2 per molecule of pyruvate.
[6] Annau and Erdoes, 1939. H. S. *257*, 111 have described an enzyme from kidney which dehydrogenates pyruvic acid and which is activated by succinic acid. Apparently YF9 and the enzyme of Annau and Erdoes are not identical.

Summary.

The cytochrome-c oxidase preparation of Keilin and Hartree contains at least three enzymes capable of oxidizing pyruvate. (1) Pyruvic ferment oxidizes pyruvic acid to CO_2 and H_2O; (2) yellow ferment 9 (lactoflavin + catalytic protein) oxidizes pyruvate to an unknown substance; (3) the aneurine ferment of Lipmann which requires B_1-pyrophosphate as a co-ferment. An enzyme (lactic ferment) has been described which catalyzes the reaction 2H + pyruvate = lactate. Both pyruvic and lactic ferments are capable of reacting with O_2 directly. Vitamin C appears to activate both enzymes. Cytochrome functions as the outlet to oxygen for yellow ferment 9 and for aneurine ferment.

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DISCUSSION

Dr. Lipmann: I have shown that the intermediate between the thiamin component and oxygen has the same prosthetic group as the *d*-amino acid oxidase of Krebs. Warburg's flavin-adenine dinucleotide did not have this intermediate at the time of last year's Congress in Zurich. I can remove this flavin by a certain procedure which I refer to in my paper at this Symposium, and I can show that this flavin is a necessary component of the system. I think that the flavin effect which Urban obtains is due to traces of flavin-

adenine present in the preparation used, and I think also that other coferments, such as the pyridine coferments, were present in his preparation in very low concentrations; in this preparation, also, the rate of oxygen consumption was very low. It is almost impossible to remove the coferment groups entirely by washing, and therefore it seems to me not quite justified to assume that the enzymes present in Urban's preparations are oxytropic and different from the known systems with cozymase and thiamin pyrophosphate, respectively, as the prosthetic groups. As to the flavin effect, I think it is due to an acceleration of the rate of reaction between the very small amount of protein-bound flavin-adenine dinucleotide and oxygen. I prefer to assume that this flavin effect is not due to a combination of flavin with a protein, forming a new enzyme, but only to an acceleration of the action of the enzymatic factors already present, as dyes are known to do in various systems.

I had observed that with my bacterial preparations dismutation of pyruvic acid is accelerated greatly by free riboflavin. But I know now that this dismutation also needs small amounts of flavin-adenine dinucleotide. In its absence the flavin is not sufficient, and from this I conclude that the flavin-adenine is the flavin that really combines with the protein, and that the free flavin does not.

Dr. Urban: It is very important that we decide at the outset whether or not we are discussing the same thing. My studies have been carried out with enzyme systems prepared from mammalian hearts. It does not follow at all that bacterial systems will be organized in their catalytic proteins and their properties in the same way as the cytochrome-c oxidase preparation from the heart.

I expected the question of traces of coferments remaining in the preparation to be raised. The only answer I am able to make is this: When we distinguish between two different reactions characterized by different respiratory quotients, the respiratory quotient is not a function of the reaction *rate*. In the second place, it will be conceded that if traces of coferments remain in the preparation (which I doubt) then the traces of coferments would function in a chain of chemical reactions, as Warburg has shown many times in his kinetic studies. It is impossible to accelerate the oxygen consumption of a reaction chain by strengthening, as it were, already strong links of a chain; you must strengthen the weakest link in order to achieve a marked acceleration, the weakest link being constituted by the traces of coferments alleged to remain in the preparation. Acceleration was achieved by increasing O_2 tension.

Dr. Lipmann: Have you ever put in the coferments and tried to see what happens?

Dr. Urban: You speak of coferments. Do you mean lactoflavin?

Dr. Lipmann: No. I mean the pyridines, flavin-adenine dinucleotide and the thiamin pyrophosphate.

Dr. Urban: I have made experiments with B_1-pyrophosphate (thiamin pyrophosphate), using pyruvic acid as substrate, and the oxygen consumption can be quantitatively calculated from the chemical analyses for pyruvic acid on the basis of two ferments operating side by side, your aneurine ferment (which uses B_1-pyrophosphate as a coferment) and my pyruvic ferment.

As to the prosthetic groups of the two enzymes which I have called lactic and pyruvic ferments, I have not yet been able to make any determinations. It is possible that we are dealing with yellow ferments. I might also say that the presence of diffusible coferments should manifest itself by the appearance of cytochrome bands in anaerobic experiments. The fact is that for long periods of time there is no spectroscopic evidence of a reaction.

Dr. Barker: Would you not think that in your cytochrome preparation you probably have carboxylase? Then when you add aneurine pyrophosphate and the pyruvate, you would expect to get decarboxylation. If the end products from pyruvate in your systems actually are CO_2 and water, would you not also have to oxidize the acetaldehyde which is formed? That is, you would have to have an acetaldehyde oxidase, too. Is there any evidence of oxidation of acetaldehyde added to your system?

Dr. Urban: It is my understanding, and I think Lipmann will bear me out in this, that in animal tissues we do not find the catalytic protein (cocarboxylase) which is necessary, in conjunction with B_1-pyrophosphate, for the decarboxylation of pyruvic acid.

Dr. Lipmann: I think that everybody agrees that there is no simple decarboxylation in animal tissue. Pyruvic acid is only broken down in the presence of a hydrogen acceptor. There are no indications of any carboxylase action in animal tissues. It is always a dehydrogenation. I think that Urban's experiment, showing that cytochrome is reduced by the addition of thiamin pyrosphosphate, is a very important one. However, I still do not feel sure it is necessary to assume that two different enzymes are present, one using thiamin pyrophosphate and the other not. Could it not be that the rate of reduction of cytochrome in the absence of thiamin pyrophosphate was so low that it could not be measured? For how long was cytochrome reduction measured in the absence of thiamin pyrophosphate? I assume that a long time was necessary.

Dr. Urban: Yes; I should say about an hour.

Dr. Lipmann: I think your rates of oxidation are so low that it might be possible that cytochrome is really reduced by the traces of prosthetic group remaining after the washing, even in the absence of added thiamin pyrophosphate.

Dr. Urban: The actual times of spectroscopic observation are so short (about 5 minutes) that any other reactions which might occur and which require hours to show up would not manifest themselves in the balance.

Dr. Stern: The question might be raised whether the system with which Urban worked was really well chosen from the experimental point of view. It is known from Green's work that lactic dehydrogenase, in contrast to succinic dehydrogenase, is a soluble enzyme. He has shown that upon repeated sedimentation of muscle preparations the lactic enzyme is removed. The O_2-uptakes reported by Urban are so low that I think he has been dealing with residual traces of the enzyme which by his twenty-one washings he almost succeeded in removing completely. To illustrate the point, the oxygen uptake for 5 milligrams dry weight of oxidase in 60 minutes is of the order of 700 to 900 mm.[3] if succinate is used, as compared with the 18 mm.[3] in Urban's experiments. Urban has mentioned that he suspected that the lactic enzyme in heart muscle may perhaps contain a flavin as the prosthetic group. But we know from Szent-Györgyi's work that lactic hydrogenase in heart muscle consist of cozymase and active protein, so there is no need at the present time to look for a further coenzyme in the prosthetic group of the heart muscle enzyme. Further, Urban adds free flavin to his preparation and observes an increase in oxygen uptake. I believe that in his experiments the lactoflavin acted as any dye of a similar potential would have acted. I do not know of any natural yellow enzyme which contains free lactoflavin in the prosthetic group. I think that in the reaction that Urban postulates here we should expect the yellow enzyme to operate in a manner similar to Straub's flavoprotein, and I do not think that Urban's experimental evidence is strong enough to postulate the formation of a yellow enzyme No. 9.

Dr. Urban: In the control determinations we are not dealing with small oxygen consumptions, but with *zero* oxygen consumption. Stern's suggestion that the lactic dehydrogenase of Green is identical with the lactic ferment which I have studied is unlikely, for the following reason: If, instead of 21 washings one stops at 11 washings, and then studies the oxygen uptake, following upon the addition of lactate to this preparation washed 11 times, one finds that there is *none*. If one analytically determines the quantities of lactic acid which have disappeared, one finds that there is no lactic acid consumption which can be measured analytically.*

It is necessary, then, to assume that we are dealing with a different ferment and one which, unlike the lactic dehydrogenase of Green, is inactive in the presence of oxygen, and which, unlike the lactic dehydrogenase of Green, requires no coferment.

Dr. Barker: Isn't the hydrogen transport of the riboflavin-protein combination much lower than when the riboflavin is phosphorylated? Considered quantitatively wouldn't there be a difference between the two compounds?

Dr. Urban: In the presence of oxygen the electron transfer by lactoflavin-catalytic protein in the experiments of Kuhn and Rudy is of the same order of magnitude as the quantity of electrons transferred by Warburg's yellow ferment.

Dr. Lipmann: The amount of free flavin Kuhn must take is, as far as I remember, about two hundred times as much as that of flavin-phosphate, in order to get the same transfer of hydrogen to oxygen.

Dr. Urban: That is correct. In order to approximate the oxygen uptake of Warburg's yellow ferment one needs perhaps as many as 50 or 100 molecules of lactoflavin per molecule of catalytic protein, but for the transfer of electrons, not to oxygen but to other redox systems, such as methylene blue, the order of magnitude is the same: about 2 molecules of lactoflavin per molecule of catalytic protein should do the same job as one molecule of yellow ferment.

Mr. Abrams: I would like to ask a question about the evidence upon which you postulate the existence of yellow ferment No. 9. You used a heart muscle preparation which is very similar to that from which Straub has isolated a flavoprotein. Straub's flavoprotein cannot be removed from the muscle by washing. Apparently, the existence of yellow ferment No. 9 is based upon the supposition that the washed heart muscle contains no flavin and that the oxygen uptake produced by adding lactoflavin is due to the combination of the latter with a specific protein to form a new yellow ferment. It seems to me, then, that your conclusions are invalidated by the fact that Straub has shown the presence of flavin in washed heart muscle.

Dr. Urban: The assumption that lactoflavin is left in the preparation is invalid because no bands of reduced cytochrome appear when the prepara-

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* A. Hahn (Z. Biol., *97*, 195, 578, 1936; *98*, 527, 1938; *99*, 614, 1939) has shown that the inhibition of lactic acid formation by oxygen in minced muscle depends on the presence of a thermostable substance which can be removed by washing. This is in agreement with our observation that lactic ferment becomes active in the presence of oxygen only after much washing.

tion and pyruvate are mixed anaerobically. In the second place, the reaction in which lactoflavin takes part can be tagged by the respiratory quotient. The only objection which could be raised against this new enzyme is the charge that the determination of the R.Q. was faulty.

Regarding the flavoprotein of Curran, Green and Straub, I was under the impression that this substance reacted with diphosphopyridine nucleotide or cozymase. I have produced some evidence which seems to justify the belief that cozymase is absent from the preparation.

Dr. Shorr: As I understand it, you postulate two different pathways for the oxidation of lactic and pyruvic acid; you have two enzyme systems dealing with these substrates in a different manner. Would it be possible to rule out the pyruvic acid pathway for the separate lactic acid system by the addition of binders such as bisulfite?

Dr. Urban: I have purposely refrained from the use of binders because one is never quite sure of the action of binders on an enzyme system. In conceiving the work I hoped that it would be possible to discover the physiological inactivators, so that the catalytic proteins could be specifically inactivated.

REACTIONS OF ASCORBIC ACID *IN VIVO*

C. G. KING

The chemical behavior of ascorbic acid (vitamin C) in comparison with other natural compounds *in vitro* is predominantly characterized by a peculiar combination of acidity and reducing action. The physiological effects of the compound have led to many suggestions regarding its chemical reactions in living cells, and in turn, the chemical investigations carried out *in vitro* have furnished preliminary evidence regarding its physiological role. Nevertheless, in a chemical sense, one is still justified in concluding that the evidence is very incomplete in regard to the major function or functions of the substance in either plants or animals.

It is impossible to review briefly a vast amount of literature and at the same time be fair to the individuals who have made important contributions, but to do so in the present case is particularly difficult because of the extremely great number of publications and the incomplete nature of the evidence that is available to support the various theories. From a research point of view, the present state of affairs may well be considered more stimulating than satisfying.

The classical observations of Wolbach (1) and others (2) on the histological effects of vitamin C deficiency have served to explain the gross symptoms that are associated with scurvy, but the chemical background for the observed tissue changes is not at all clear. Neither have the physiological or functional effects of vitamin C deficiency been correlated with specific chemical reactions of the vitamin (3, 4), except perhaps in the recent work of Ecker and associates (5), in relation to the blood complement factor. The finding by Bonner and Bonner (6) that ascorbic acid functions as a specific plant growth stimulant has opened another interesting field where there is no chemical basis for explaining an important biological phenomenon.

The Distribution and State of the Vitamin in Tissues

The almost universal occurrence of ascorbic acid in relatively high concentrations in living plants and animals, and the rapidity of its changes in concentration with increased or decreased physiological activity, together with its sensitivity toward oxidizing agents, certainly argues strongly for a biochemical role of major importance in the functioning of nearly all cells.

The question of whether the acid occurs or reacts in a conjugated form with proteins has not been answered satisfactorily. McHenry has been particularly active in presenting evidence for the occurrence of such protein conjugates (7). Other investigators (8, 9) have questioned the correctness of McHenry's interpretation (the occurrence of ascorbic acid in a conjugated form that resists extraction with cold acids) largely on the basis that the technique used may have permitted partial oxidation of the vitamin, or that the washing of ground tissue may not have been sufficiently thorough. Similar disagreement remains in regard to the occurrence of the vitamin in significant amounts in the reversibly oxidized state: a number of investigators hold to the view that the oxidized form as shown by analysis has represented a product that was formed apart from the normal reactions within the living cells. The rapidity with which nearly all of the ascorbate in animal and plant tissues can be extracted with cold acid media or hot neutral solutions when oxidation is prevented, affords only preliminary evidence of the occurrence of the acid chiefly as a freely diffusible acid or salt. One cannot be certain that weakly conjugated products have not been dissociated by the reagents used for extraction. Diffusion from microtome tissue slices (10), *Chlorella* cells (11), the common algae (12), etc., permits nearly quantitative recoveries in a short time, however.

The work of Bourne (13), Leblond and Giroud (14) affords evidence of the concentration of the vitamin in granules adjacent to the Golgi apparatus and mitochondria, and perhaps on the surface of various lipid phases in the cell, but the staining technique may not be sufficiently delicate to show whether the granular silver deposits represent separate phases of the combined vitamin, or possibly local areas of high concentration in solution.

The observations of Hopkins and Morgan (15) may be interpreted as evidence for at least sufficient conjugation to form a completed enzyme system, even though the quantity combined at any given time might be relatively small. The suggestion that liver esterase may consist of a protein conjugated with ascorbic acid (16) has not been verified. Harrer's findings (17) were not in accord with the latter theory; scorbutic animals did not show a significantly lowered esterase value in their tissues.

Type Reactions of Ascorbic Acid

Acid reactions.

The acidic dissociation of ascorbic acid involves the enolic groups on carbons 2 and 3, as shown by the structural studies of Karrer, Haworth, von Euler, Reichstein, Micheel and others (18). The dissociation constant [Birch and Harris (19), $pKa = 4.17$; $pKa_2 = 11.57$; Ball (20), $pK =$

4.21; Kumler and Daniels (21), pK = 4.12] makes it evident that in animal tissues, and in most plant tissues as well, the acid would be present almost entirely as the anion of a salt or as a non-dissociated organic complex. The lactone ring does not open in physiological pH ranges, in which respect the behavior of ascorbic acid differs from the more common sugar-acid lactones except those with an analogous dienol grouping on carbons 2 and 3.

Many organo-metallic complexes of ascorbic acid with metal ions such as those with iron, manganese and copper salts have been prepared in the laboratory, but one may well question whether the complexes thus prepared occur in nature or have any biological significance.

Hawley observed a marked influence of acid-base balance upon ascorbic acid excretion clinically (22), but we did not find an analogous condition in the rat (23) when the pH of the urine was varied from 6.3 to 8.3 by feeding NH_4Cl and $NaHCO_3$.

Oxidation-reduction reactions.

The greatest chemical interest in ascorbic acid has been associated with its oxidation-reduction reactions. Ball (20) has recently recorded a series of measurements of the oxidation-reduction potential of the ascorbic-dehydroascorbic acid system and has cited references to the earlier papers of Borsook (24) and a number of other investigators. In order to obtain satisfactory potential measurements at the electrode surface it was necessary to have a mediator present in the solution, such as one of the dyestuffs, and to work at a pH low enough to avoid interference due to decomposition of the unstable dehydroascorbic acid. The findings of Borsook and associates were of special interest in relation to the decomposition rate and products of dehydroascorbic acid. No satisfactory scheme of intermediate reactions has been devised to explain the intermediate steps between the initial dehydroascorbic acid and the final oxidation products, oxalic and l-threonic acids.

The rate of oxidation of ascorbic acid in aqueous solutions, both by air and by many dyestuffs, is extremely rapid compared to the rates for other organic substances. For example, the sulfhydryl compounds that reduce dehydroascorbic acid are less sensitive to oxidation than the acid itself under commonly observed laboratory conditions.

An outstanding characteristic of ascorbic acid is its sensitivity to oxidation and decomposition under the influence of catalysts that occur in all living cells. The practical significance of the last point is very great, because it accounts for the fact that vitamin C can be found only in fresh foods or products that have been preserved under special conditions to avoid oxidation. In many vegetables the rate of loss is fairly rapid even when stored with dry ice. Unless the acidity is high, crushed or sliced plant tissues lose their ascorbic acid content within a few minutes, and animal tissues commonly lose from 10 to 50 per cent of their ascorbic acid content per day.

It is evident that many organic compounds might reduce the reversibly oxidized form of ascorbic acid, but nearly all of the experimental work reported in the literature has dealt solely with the reducing action of sulfhydryl groups, particularly with H_2S, cysteine, glutathione and protein-SH.

Condensation and esterification.

Ascorbic acid and dehydroascorbic acid both exhibit the usual reactions that would be expected for the respective primary and secondary alcohol, dienol, and adjacent diketone groups. Yet there is no clear evidence that such groups are in any way reactive in vivo except as noted above in relation to acidic dissociation and oxidation-reduction reactions. There is no evidence, for example, that phosphorylation or other types of esterification occur, or that ethers are formed in vivo by reactions of the enolic groups.

Dehydroascorbic acid can serve as an oxidizing agent for the conversion of alpha amino acids to aldehydes and ammonia in vitro, as shown by Abderhalden and others (25), but there is no evidence that the reaction is of importance biologically. The nearest approach in this direction is probably the work of Euler, Karrer and Zehender (26) in demonstrating the deamidation of leucine. It would be of distinct interest to see their work continued in relation to in vivo reactions.

Sensitivity to Biological Oxidation Systems

Molecular oxygen and metal ion catalysts.

Long before vitamin C had been identified as a single chemical compound (18) its sensitivity to copper-catalyzed oxidation was widely recognized (27, 28). Barron's work (29), however, was especially significant in showing that pure ascorbic acid in aqueous solution was not autoxidized within common physiological pH ranges (<7.6). The inhibiting or accelerating effects of anions on copper ion catalysis was demonstrated by Lyman and associates (30).

Many of the higher valence metal ions such as Fe^{+++}, Cu^{++}, Hg^{++} and Mn^{+++} oxidize ascorbic acid directly and rapidly, but over a wide pH range the copper ion is remarkably more effective than the others as a catalyst for the reaction with molecular oxygen. The effect that combining copper with known groups would have upon its catalytic activity affords an interesting

field of study. From such data one might reasonably hope to obtain valuable leads concerning the nature of the active groups in native catalysts. Most of the sulfur-containing complexes such as those with sulfide, thiocyanate, xanthate, thiocarbamate, cysteine, glutathione, and thiourea (31, 32) are apparently inactive. Other combinations remain very active. Copper-amide-biuret, for example, was found by Stotz *et al.* (31) to be atom-for-atom as active as the Cu^{++} ion. The one copper-porphyrin compound examined in our laboratory was also very active.

Copper-protein catalysts.

Copper ion added to protein solutions was found to be inactivated at low concentrations, varying widely with the nature of the protein, but when larger amounts of copper were added the copper-proteinates remained active and showed properties very much like some of the native plant enzymes that had been described in the literature; *i.e.*, the synthetic preparations exhibited similar optimum pH values, were thermolabile, and were essentially identical in their sensitivity to organic and inorganic inhibitors. The only difference observed was the greater ease of removing the added copper by simple dialysis. A number of the inhibitors used were not effective against iron catalysis; so far as is known they are specific for copper in the concentrations used. In view of the many similarities found between the copper-protein preparations and the partially purified plant enzymes, particularly the sensitivity to copper poisons, and the fact that the enzyme preparations available at that time contained enough copper to account for their catalytic activity, it was suggested that the widely distributed plant protein catalysts, such as those described by Szent-Györgyi (33), Srinivasan (35), and Tauber and Kleiner (34), were, in large part at least, compounds of

TABLE I

Effect of copper inhibitors

Ascorbic acid, 0.01 mM; $T = 37°$; pH $= 6.0 \pm 0.1$.

Inhibitor	Catalysts						
	Cu, 3×10^{-4} mM	Cu-albumin, 2×10^{-4} mM Cu	Cu-gelatin, 6×10^{-5} mM Cu	Nicotine-hemo-chromogen, 3×10^{-4} mM hemin	Cauliflower press-juice, 5×10^{-5} mM Cu	Squash "oxidase," 3×10^{-5} mM Cu	Cabbage juice
	Rate of O_2 consumption, mm³. O_2 per hr.						
None	420	260	240	320	220	290	90
	Per cent inhibition						
Diethyldithiocarbamate, 3×10^{-3} mM*	100	96	98	0	76	100	68
8-Hydroxyquinoline, 7×10^{-3} mM	99	92	96	0	42	94	62
Pyridine, 1 mM, KCNS, 1 mM	92	90	98	−37†	84	85	50
Sodium cyanide, 0.04 mM	98	95	97	48	88	95	56
Potassium ferrocyanide, 0.025 mM	93	87	90	0	58	89	32
Potassium ethyl xanthate, 0.06 mM	95	78	88	0	83	97	68
Sodium sulfide, 0.025 mM	98	86	85	8	91	96	35

* The quantities of copper, ascorbic acid, and inhibitors recorded in this table represent the total amounts in the 3 cc. volume of reactants. The total amount of albumin and gelatin in 3 cc. was 10 mg.
† 37 per cent increase in O_2 consumption rate.
(J. Biol. Chem., *119*, 511 (1937)).

similar nature (*i.e.* essentially copper-protein compounds) (31). We have obtained purified preparations from a number of different plant sources, all containing copper and showing the same sensitivity toward copper inhibitors, but we have not yet succeeded in obtaining a pure crystalline enzyme of the "ascorbic acid oxidase" type. Szent-Györgyi (36) has recently reported the preparation of an enzyme from cucumber juice that was more active than copper ion on the basis of its copper content, indicating either that the activity was not dependent upon copper or that the activity of the copper was enhanced by the protein component. Clear-cut evidence of the effect of the protein carrier upon the substrate specificity of copper toward ascorbic acid and catechol was shown by McCarthy and associates (32). The addition of copper ion to inactivated cucumber oxidase and inactivated potato polyphenol oxidase (the latter having been shown by Kubowitz (37) to be a copper-protein catalyst), was followed by restoration of catalytic activity in each case, without a loss of substrate specificity when retested with ascorbic acid and catechol. The two substrates are also very different in their sensitivity to simple copper ion catalysis, the catechol being relatively inert. It seems almost certain that the plant catalysts described as "hexuronic acid" or "ascorbic acid" oxidase, including those described recently by Zilva and associates (38), were largely protein-copper complexes, in some cases mixed with varying amounts of quinone-forming material and peroxidase.

Hemochromogens.

The hemochromogens can also serve as oxidative catalysts for ascorbic acid, as shown clearly by Barron and associates (29), and by other investigators. The activity of cytochrome-c and cytochrome oxidase for ascorbic acid oxidation was shown by Stotz, *et al.* (39), Keilin and Hartree (40), and Karrer, Euler and Hellstrom (41). From the inhibition characteristics of animal tissues (39) it appeared that hemochromogen type catalysts were chiefly involved in the catalysis of ascorbic acid oxidation, perhaps through the cytochrome oxidase system, as suggested by Stotz and others. In recent demonstrations of the marked effect of copper (but not iron) intake on the cytochrome oxidase in rat tissues, however, Schultze (42) observed that the cytochrome oxidase activity varied without any apparent correlation with the capacity of the tissues for catalyzing the oxidation of ascorbic acid. Neither was there any evidence of a relation between the copper in the tissues and their capacity to catalyze ascorbic acid oxidation. A high-iron, low-copper diet led to a moderate increase in this capacity, however, indicative again of a hemochromogen effect, even though the tissues were low in cytochrome oxidase.

Lemberg and associates (43) have published a series of observations on the role of ascorbic acid as one of the reactants, together with oxygen, in bringing about the formation of bile pigments from oxyhemoglobin. The coupled oxidation resulted in opening the hematin ring without loss of the combined iron. These observations are at least of marked interest in relation to the origin of hematin decomposition products. The physiological significance of the reactions (and their possible relation to other oxidative reactions) will no doubt become more clear with continued investigation. The formation of hydrogen peroxide from ascorbic acid did not appear to be an essential part of the reaction.

Quinone type oxidants.

Ascorbic acid is oxidized to dehydroascorbic acid very rapidly by many quinones, quinonimides, indophenols, flavones, and other compounds of the quinonoid type. Several different specific reaction systems have now been identified in which the quinonoid substance acts as a very rapid hydrogen carrier between ascorbic acid and molecular oxygen, through the agency of an oxidase to promote quinone formation. Kubowitz, with potato polyphenol oxidase and catechol (37), Keilin and Mann, with mushroom oxidase and catechol (44) and lacquer latex oxidase with *p*-phenylene diamine (45), and Dalton and Nelson, with another mushroom oxidase and catechol (46) have shown striking examples of such carrier reactions between ascorbic acid and molecular oxygen. Less highly purified enzyme preparations that give rise to quinone formation upon exposure to air, and hence oxidize ascorbic acid in a similar fashion, have been described by many investigators (38, 47, 48, 49). In view of the high concentration of ascorbic acid in nearly all active plant tissues and the widespread occurrence of the quinone-forming oxidases with their respective substrates, it appears that the above type-reaction is one of importance in plant physiology, irrespective of whether one considers the major role of ascorbic acid to be (a) respiratory, (b) protective against the accumulation of excessive amounts of oxidants, such as quinones (or adrenochrome in animal tissue), (c) regulative in the sense of maintaining enzymes such as the proteases, phosphatases, etc. in a proper state of activity, or (d) some other function.

Relatively few papers have considered the reactions between ascorbic acid and the flavin carriers. Hopkins (50) and Hand, *et al.* (51) have demonstrated the rapidity with which ascorbic acid is oxidized by flavin when a solution containing both (or milk) is exposed to light, but there

is little evidence concerning the possible significance of such a reaction in tissues such as the skin or eyes.

Peroxides and peroxidases.

Hydrogen peroxide, alone or in the presence of peroxidase, is a relatively slow oxidant for ascorbic acid (49). Most of the data relative to ascorbic acid oxidation through the role of peroxidases have been obtained in the presence of quinone forming materials, in which the latter served as carrier oxidants. The accumulation of H_2O_2 in parallel with its slow reaction with the remaining free ascorbic acid causes considerable difficulty when one attempts to correlate oxygen uptake with the conversion of ascorbic acid to dehydroascorbic acid.

The Reduction of Dehydroascorbic Acid, and the Possibility of a Carrier Role

Dehydroascorbic acid can be reduced to ascorbic acid by hydrogen sulfide with nearly quantitative recoveries in the pH range of 3 to 4. The reduction with glutathione and protein sulfhydryls is less rapid and less complete, however, especially when there is not a large excess of the reductant. The slowness and incompleteness with which recognized animal tissue reductants act upon dehydroascorbic acid constitutes one of the most serious grounds for questioning whether the vitamin serves as a major respiratory carrier in animal tissues.

Schultze and associates (52) have shown that when glutathione is added to dehydroascorbic acid in a ratio approximating that found in animal tissues, the rate of reduction is relatively slow and the reaction is far from complete. In tissue brei the reduction of dehydroascorbic acid was apparently due almost entirely to glutathione and protein sulfhydryl. The presence of an excess of ascorbic acid in the solution with dehydroascorbic acid, however, tended to prevent the reduction of

relatively small amounts of dehydroascorbic acid. When iodoacetate was present, or when alloxan or arsenite oxidation was used to block the available sulfhydryl groups in both dialyzed and non-dialyzed guinea pig tissue, there was very little reduction of dehydroascorbic acid. No evidence could be found for enzymic reduction of dehydroascorbic acid in either guinea pig or rat tissue. The examples of sulfhydryl reduction of dehydroascorbic acid recorded in the literature have been based upon the use of an excess of the reductant and an unphysiological ratio of oxidized-to-reduced ascorbic acid (15, 24).

More recently, Schultze and associates (in press) have studied the possible reaction between coenzyme I and dehydroascorbic acid, but no appreciable reduction could be observed, either with the purified (50 per cent), reduced diphosphopyridine nucleotide alone, or in the presence of the water-soluble, dialyzed constituents of minced rat liver or muscle. Euler reported (53) that Adler and Günther had obtained results indicating that such a reaction takes place, but their results have not been published in detail. In the experiments of Schultze, et al. it was further observed that ascorbic acid alone or ascorbic acid with glutathione did not serve as a hydrogen carrier between the glucose dehydrogenase system and hemochromogens.

Hopkins and Morgan (15) presented evidence for the presence of an enzyme in cauliflower juice that catalyzed the reduction of dehydroascorbic acid by glutathione, but Kertesz (54) could not duplicate their results. Crook and Hopkins repeated the original observations, however (55), and placed special emphasis upon the instability of the enzyme, both in the intact plant under market conditions and in the press juice.

The work of Ecker and associates (56), in which a correlation between ascorbic acid in the blood of guinea pigs and the maintenance of normal complement activity was noted, has been

TABLE II

Correlation of scurvy score with O_2 consumption rate of liver

No. of animals	Scurvy score	Average Q_{O_2}	Per cent increase in Q_{O_2}*
41	0 (Normal)	4.5	
17	5-11	5.8	29
18	12-15	6.1	36
9	16-21	6.3	40

* Q_{CO_2} increases also so that CO_2/O_2 and the true R.Q. remain unchanged.

(J. Biol. Chem., *120*, 129 (1937)).

verified in another laboratory (57). The primary change involved was thought to be associated with a reversibly oxidized serum protein. The authors believe that ascorbic acid is the agent most concerned with maintaining the complement in a reduced, active state, but that other reducing agents such as glutathione can serve to maintain a subnormal state of activity.

In the study of respiration changes in tissue slices coincident with scurvy, Stotz and associates (58) observed an average rise of 40 per cent in the Q_{O_2} values for scorbutic guinea pig livers, rather than a drop in value as one might expect if the vitamin were serving as a respiratory carrier. Aerobic and anaerobic glycolysis and respiratory quotient values remained essentially unchanged. McHenry and associates have observed a similar rise in the basal metabolism of scorbutic guinea pigs (18th day) when the animals were studied by the paired feeding technique (59) (+19, +19, and +20 per cent in 3 different experiments). Six earlier papers by other authors had reported decreases in oxygen consumption with the onset of scurvy, and two papers had reported increases.

Precursors of ascorbic acid.

It is clearly evident that nearly all growing plants and nearly all animals, including the developing chick embryo (60, 18), can synthesize ascorbic acid from common nutrients and maintain normal tissue concentrations. The nature of

TABLE III

Reduction of dehydroascorbic acid by liver, heat-coagulated liver, and glutathione in presence of ascorbic acid

Time 30 minutes; anaerobic, 37.5°, pH 7.4. Total ascorbic + dehydroascorbic acid, 2 mg.

Reducing system	Ascorbic acid added	Dehydroascorbic acid added	Added dehydroascorbic acid reduced
	mg.	*mg.*	*per cent*
Liver, brei, 0.8 gm.	1.80	0.20	0
	0	0.20	44.5
	1.40	0.60	20.0
	0	0.60	32.5
	1.00	1.00	28.0
	0	1.00	28.2
Heat-coagulated liver, 0.8 gm.	1.76	0.24	0
	0	0.24	30.0
	1.64	0.36	2.8
	0	0.36	28.4
	1.52	0.48	16.7
	0	0.48	26.2
Glutathione, 3 mg.	1.76	0.24	4.2
	0	0.24	34.7
	1.64	0.36	16.6
	0	0.36	30.5
	1.52	0.48	18.4
	0	0.48	30.8
	1.40	0.60	35.0
	0	0.60	30.0
	1.00	1.00	28.0
	0	1.00	26.6
	0.60	1.40	28.8
	0	1.40	25.0

the precursor substance or substances and the site of tissue synthesis are still unsolved but interesting problems.

The early experiments in which special emphasis was placed upon mannose as a precursor could not be verified in other laboratories.

The observations of Reid (61) and Bonner and Bonner (6) point toward the role of glucose as a possible precursor of ascorbic acid in plants. The importance of light for the synthesis of ascorbic acid after the food reserves of the seed were depleted by young plants, was shown strikingly by Reid.

The study of ascorbic acid synthesis and excretion in rats has given interesting leads toward some of the factors that are involved in its origin in animal tissues. Musulin, Longenecker, Tully and King (62) have found that a number of natural foods cause an average excretion rate of about 2 mg. per day, in contrast to an excretion rate of about 0.3 mg. per day on a diet of evaporated milk or Dryco. Fractionation of the active material from foodstuffs led to the finding that a great many aromatic and aliphatic ketones and related compounds could cause average excretions in the range of 5 to 15 mg. per day—quantities that obviously involved an accelerated rate of synthesis as well as increased excretion, because the quantity excreted daily over long periods greatly exceeded the total quantity in the rat's body at any one time. During the periods of accelerated or depressed excretion there was very little change in the ascorbic acid content of the blood or tissues. The technique of study will probably be of value in that it provides a method of causing controlled and regular variations in the rate of synthesis in a standard laboratory animal, through the feeding or injection of known compounds.

TABLE IV

Excretion of vitamin C by rats following ingestion of cyclic ketones and alcohols

Substance fed	Quantity fed per day	Average daily excretion		Maximum average excretion
		1st to 4th day	4th to 8th day	
	mg.	*mg.*	*mg.*	*mg.*
Cyclic ketones				
d-Carvone	100	6.0	12.8	16.5
"	50	3.0	6.2	7.6
"	20	2.2	3.6	5.2
l-Carvone	100	4.4	6.2	8.0
Isophorone	100	5.4	8.1	10.8
"	20	3.2	6.8	7.5
dl-Piperitone	100	4.2	6.2	8.1
"	20	2.5	3.6	4.1
Thujone	20	1.4	3.8	4.9
Pulegone	100	1.5	1.7	2.0
Camphor	20	1.9	2.2	2.6
Cyclohexanone	100	0.9	1.1	1.5
Cyclopentanone	100	0.8	1.2	1.5
Acetophenone	50	0.8	1.0	1.1
Dihydroandrosterone	20	0.2	0.3	0.3
Cholestenone	20	0.3	0.4	0.5
Kojic acid	25	0.3	0.4	0.4
Quinone	20	0.5	0.5	0.7
Cyclic alcohols				
Menthol	20	0.6	1.0	1.5
Isoborneol	20	0.7	1.6	1.8
Cineole	25	0.7	2.1	2.3

Summary

A fairly extensive amount of experimental work reported in the literature tends to support the theory that ascorbic acid can serve as a respiratory catalyst in certain plants. Oxidative catalysts that are very effective either directly toward oxygen, or indirectly through quinone carriers are very wide-spread in plant tissues. The nature of rapid reducing systems has been established less clearly, but Hopkins' work points strongly toward the existence of a catalyst linking dehydroascorbic acid with the sulfhydryl group in glutathione. The results thus far do not definitely establish the viewpoint, however, that the major function of ascorbic acid in plants is that of a respiratory or hydrogen-transfer agent.

In animal tissues the chemical role of the vitamin is even less clear. It is moderately sensitive to recognized oxidative catalysts such as cytochrome-c and the simple hemochromogens, and the reversible oxidation product reacts in some degree with reducing agents of the sulfhydryl type, but there is still little evidence to indicate that it serves as an important hydrogen carrier. The evidence is more negative than positive in regard to its postulated respiratory role in animal tissues. Three points are particularly difficult to explain on the assumption that it serves primarily as a carrier: (a) The living scorbutic animal shows an increased rather than a decreased respiratory rate when compared with normal animals: (b) tissue slices show a similar phenomenon—an increased rather than a decreased respiratory rate coincident with the onset of scurvy; and (c) the addition of ascorbic acid to normal or scorbutic tissue slices does not increase the rate of respiration. Neither is there a significant change in the rate of aerobic or anaerobic glycolysis or in the respiratory quotient of tissue slices from scorbutic animals. In relation to the respiratory reactions in animal tissues, it seems more logical at the present time to view ascorbic acid as a regulating and protective agent, perhaps exerting an effect upon other more effective hydrogen carriers and other important enzyme systems, rather than functioning as a major carrier itself.

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DISCUSSION

Dr. Barron: With reference to the effect of ions on the oxidation of ascorbic acid in heavy metal free buffer solutions, I may add one experience which we had, and that is the effect of acetate. Acetate seems to induce the autoxidation of ascorbic acid to a slight degree.

We have had very much the same experience as King had on the role of ascorbic acid in oxidation enzymatic processes. The experiments were designed to agree with thermodynamic possibilities and to avoid destruction of the reversible oxidized ascorbic acid. Working at 37° C., pH 5.0, to test the role of ascorbic acid as reductant, we chose fumaric acid and the enzymic succinic dehydrogenase; to test the role of dehydroascorbic acid as oxidant we chose lactic acid and hydroxyoxidase. No oxidation of lactic acid could be found in the presence of the activating enzyme and lactic acid.

The same negative results were observed when we chose ascorbic acid as the reducing agent. We did quite a number of experiments and in no case did ascorbic acid act as a reductant of fumaric acid. So we have here two cases where ascorbic acid might act as electron mediator in enzymatic sluggish oxidation-reduction systems. In no case had ascorbic acid such an action.

There is another objection to the possibility of ascorbic acid acting as an oxidation or reduction agent in animal tissues, and that objection is the fact shown by Würmser and ourselves that the reversible oxidized ascorbic acid is destroyed rather quickly from pH 5 up.

On studying the degree of reduction of the dehydroascorbic acid we found that we could find 100 per cent reduction only at pH 5. The degree of reduction of dehydroascorbic acid could be reduced. This seems to me a serious objection to the possibility of ascorbic acid acting as an electron transfer in animal tissues, since in general the pH of animal tissues is around neutrality.

I quite agree with King's comments on the effect of glutathione in animal tissues. I might add further that in experiments where we added small concentrations of glutathione, *i.e.* similar to those found in cells, we found no evidence that glutathione could reduce ascorbic action. At such concentrations glutathione inhibited the catalytic action of copper on the oxidation of ascorbic acid.

We found the same thing when we used small amounts of copper and large amounts of protein. There was an apparent discrepancy between our paper where we showed that protein would inhibit copper catalysis and the paper of Stotz and King where they found that copper proteins act as catalysts. The discrepancy is only apparent, because King used large amounts of copper and we used small amounts of copper and large amounts of protein in order to show that the higher catalytic effect of copper in our experiment was due to the fact that the copper was in the ionic condition. As long as we diminished the concentration of copper as ionic copper by combining it with amino acids or proteins and forming in that way un-ionized copper complexes, we found we could inhibit the action of ascorbic acid.

I would like to ask King, if he assumes a respiratory role for ascorbic acid in plants, why is it that very few plants have this so called ascorbic acid oxidation enzyme? I think in one of our papers dealing with ascorbic acid in biological fluids and in the study of fluids obtained from plants, there were only four where ascorbic acid could be oxidized by the juice of the plants. In all the rest ascorbic acid was protected from oxidation and always remained in a reduced condition; that is to say, a number of plants having no protective action for the oxidation of ascorbic acid use this system in respiration, and other plants, which have this protective action, do not use ascorbic acid in respiration.

Dr. King: I am glad that you pointed out the fact that both laboratories are in complete accord on the protein effect. Differences in proteins may be striking too, in addition to the effect of concentration. In our work with gelatin and albumin (albumin has a high sulfur content compared to

gelatin), gelatin would inhibit relatively small amounts of copper, but albumin was very effective.

With regard to the respiratory role in plants, I feel that two arguments favor considering ascorbic acid as a possible carrier. First is Hopkins and Morgan's work in which they have interpreted their results as indicating a specific enzyme for the reaction between glutathione and dehydroascorbic acid. We have not found results of that kind in our experience, but we have not studied their specific reaction conditions with sufficient care that I would want to question the validity of their results. In other words, we feel that Hopkins and Morgan's observations give a strong indication of such a mechanism and I do not like to question their work unless one has strong experimental evidence to the contrary.

The second point which makes me more receptive to the concept of some respiratory function of ascorbic acid in plants is the fact that plants are much more heavily loaded with quinone-forming materials than are animal tissues, and therefore they have a more adequate oxidative system to which ascorbic acid is very sensitive. However, I do not believe that it is established beyond question that ascorbic acid is a respiratory agent, even in plants.

Dr. Barron: Hopkins and Morgan found that glutathione and ascorbic acid can react in plant tissues, but they used large concentrations of glutathione as compared with the ascorbic acid. That is why I object to trying to relate their experiments to plant respiration. As you have pointed out, there is very little glutathione in plants and large amount of ascorbic acid. If we are going to do a physiological experiment we have to use the same ratios.

Dr. King: I agree that it is improbable that it acts as a major carrying agent; but possibly it is a minor respiratory agent. I agree, too, that the experiments in Hopkins' laboratory were not physiological in the sense of ratio. With ratios as we have studied them in other systems, we have had nothing in our own experience, and I know of nothing in the literature, which would support that point of view.

Dr. Salomon: I would like to mention with reference to the facts stated by King, some unpublished experiments of my own concerning the oxidation of cysteine and ascorbic acid. I compared, in a study of copper catalysis, the catalytic power of copper protoporphyrin with that of an equivalent amount of copper on the oxidation of cysteine under metal-free conditions. Whereas the catalytic activity of copper on the oxidation of cysteine reaches a well defined maximum at pH 7.8 at a temperature of 30° C., the catalytic activity of the copper protoporphyrin is the same in the system mentioned between pH 7.2 and pH 7.8.

The catalytic activity (according to Warburg the efficiency of the catalyst as expressed in terms of O_2/mg. Cu $\times$ hours) of the two agents was of the same order of magnitude.

The oxidation of ascorbic acid is in a definite, although not linear, relation to the amount of copper present. The effective copper concentration has been found by Barron to be between 10^{-2} and 10^{-4} mg. We found that the velocity of ascorbic acid oxidation can be used for the determination of copper in the range from 3 to 8 micrograms, which is biologically most interesting. One of the main advantages of the method consists in the fact that ascorbic acid is not sensitive to the presence of manganese, iron, nickel, or cobalt.

Dr. Müller: Have you studied the elimination of ascorbic acid in rats when the animal was hypophysectomized? Phillips of Cornell Medical School has worked with hypophysectomized rats which showed all the outward signs of scurvy, when given a special diet. This he thinks he has been able to overcome partially by the addition of ascorbic acid to their diet.

Dr. King: We have not worked with hypophysectomized animals at all, but we can report that the composition of the diet has a great deal to do with the response on feeding these different compounds. One can maintain an animal on experimental diets for long periods without having within the animal a "normal" capacity to respond to these organic compounds, and without a marked change in the ascorbic acid content of its tissues. That is rather baffling at the present time, but I am sure that it is true.

Dr. Hogness: How was the test for cytochrome oxidase made in your experiments on the feeding of a high copper diet to rats—those which showed that such a diet brought about an increase in cytochrome oxidase activity but no increase in the rate of ascorbic acid oxidation? There is the danger that, if hydroquinone were used as the substrate, its oxidation could be catalyzed directly by the copper ion, without the intervention of the cytochrome-c and its oxidase.

Dr. King: Schultze has used the method which Stotz and his associates have recently worked out and has obtained very good results. That is, he checked with the method that you have used in measuring cytochrome oxidase as a separate phenomenon, working with an excess of purified cytochrome-c, and in the presence of semicarbazide to combine with the quinone formed. So I am reasonably sure that copper as such could not have been the controlling agent in the system studied.

Dr. Barker: Is there any possibility of some of the compounds fed being mildly toxic? Not being of value to the animal body, perhaps they were excreted in a conjugated form which might

show up in the titration method of analysis for the ascorbic acid. I am thinking especially of the cyclic compounds. You were not able to obtain anti-scorbutic activity of the urine except in the higher excretion levels, as I remember.

Dr. King: We have never made assays when the excretion rate was below two milligrams per day.

Dr. Ball: With regard to the role of ascorbic acid as a cyclic mediator in biological oxidations I should like to mention the following observations:

$$
\begin{array}{cccc}
\text{O} & \text{O} & \text{O} & \text{O} \\
\parallel & \parallel & \parallel & \parallel \\
\text{C}{-} & \text{C}{-} & \text{COH} & \text{COH} \\
| & | & | & | \\
\text{HOC} & \text{O}{=}\text{C} & \text{O}{=}\text{C} & \text{HOC} \\
\parallel\;\text{O} \rightarrow & |\;\;\text{O} \rightarrow & | & \parallel \rightarrow \\
\text{HOC} & \text{O}{=}\text{C} & \text{O}{=}\text{C} & \text{HOC} \\
| & | & | & | \\
\text{HC}{-} & \text{HC}{-} & \text{HCOH} & \text{HCOH} \\
| & | & | & | \\
\text{HOCH} & \text{HOCH} & \text{HOCH} & \text{HOCH} \\
| & | & | & | \\
\text{H}_2\text{COH} & \text{H}_2\text{COH} & \text{H}_2\text{COH} & \text{H}_2\text{COH} \\
\text{I} & \text{II} & \text{III} & \text{IV}
\end{array}
$$
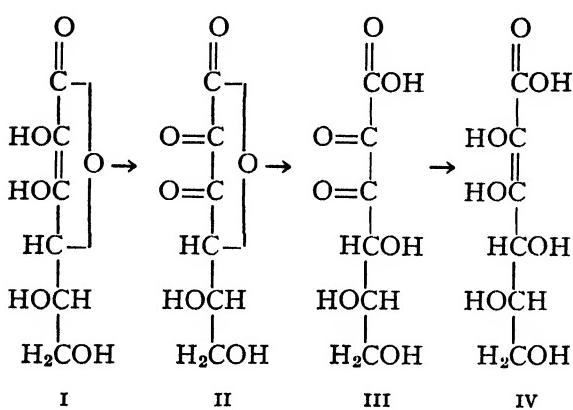
As pointed out by King the possibility that ascorbic acid (I) acts as the reductant of a reversible oxidation-reduction system whose oxidant is dehydroascorbic acid (II) appears remote in animal tissues. Dehydroascorbic acid at body pH and temperature is an unstable compound with a half-life of about two minutes. It apparently rapidly undergoes transformation into compound (III) by opening of the lactone ring. This product, however, may then conceivably undergo a reduction to produce the enol form (IV) of 2-keto-1-gulonic acid which may be converted into ascorbic acid by the removal of water to reform the lactone structure. Such a chain of reactions might thus represent a way that ascorbic acid can act as a mediator in biological oxidations. This possibility can, however, be tested by determining the anti-scorbutic properties of 2-keto-1-gulonic acid. This compound was therefore synthesized by the method of Reichstein and Grussner. When tested on guinea pigs it was found to be devoid of anti-

scorbutic properties. This compound can therefore apparently not be considered as a precursor of ascorbic acid in the animal organism.

Dr. Hogness: Coming back to the question of the test for cytochrome oxidase, I don't think that the test which Stotz and I proposed, using hydroquinone, would preclude any action by copper, because hydroquinone could be oxidized through the copper ion as a catalyst. I don't recall any part of the test which would rule that out. So I still consider it is a possibility. The better test would be to use reduced cytochrome-c rather than the hydroquinone as the substrate.

Dr. King: According to our experience, if the increased rate of oxidation of hydroquinone had been primarily due to copper, there would have been a much more rapid oxidation of ascorbic acid (relative to hydroquinone). The oxidation of hydroquinone (compared with ascorbic acid) is catalyzed more effectively by cytochrome oxidase than by copper ion.

I would like to introduce into the discussion a communication from Peugnet and his collaborator, Urban, summarizing some of their observations in relation to ascorbic acid.

Dr. Peugnet: Investigations carried on by Urban and me have brought out the following points which may be of interest in relation to the function of vitamin C.

(1) Ascorbic acid exerts a powerful beat-strengthening action on the ventricle of the frog heart.

(2) Ascorbic acid accelerates the beat frequency of the abnormally slow pace-maker with little or no effect upon the normal rate, in which respect it differs distinctly from adrenalin.

(3) Ascorbic acid frequently increases the diastolic "tonus", but this action has not been consistently reproducible.

(4) Inotropic effects are obtainable in concentrations as low as 0.05 mg. per 100 cc.

(5) The ascorbic acid action is independent of and additive with that of perfusate glucose.

(6) The action is not due to atropine-like vagus paralysis, nor to removal of inhibitory action of perfusate potassium, nor to calcium contamination of the vitamin C, nor to pH effects.

(7) Ascorbic acid oxidized to dehydroascorbic acid by means of iodine is inactive.

(8) The presence of copper is essential to the action of ascorbic acid on the frog heart.

TYROSINASE

J. M. Nelson

The phenolase, tyrosinase, occurs widely in nature, especially in the plant kingdom. In the natural state it is usually found accompanied by other phenolases, peroxidases, catalases, etc., many of which influence its enzymic action. It is distinguished from the other phenolases by its ability to catalyze two essentially different aerobic oxidations, the insertion of a hydroxyl group into certain monohydric phenols *ortho* to the one already present, and the oxidation of certain *o*-dihydric phenols to their corresponding *o*-quinones.

The two substrates which have been most generally used for studying the mechanisms of these two types of reactions are *p*-cresol for the monohydric oxidation and catechol for the *o*-dihydric oxidation. Since the oxidation of the *o*-dihydric phenols seems at present to be the simpler, it will be taken up first.

When catechol is oxidized by means of tyrosinase two atoms of oxygen are consumed per mole of catechol (1). Although only one atom is required, stoichiometrically, for the formation of *o*-benzoquinone, the normal product of primary oxidation, the question naturally arises as to what becomes of the second atom of oxygen involved in the oxidation.

Due to the fact that in the autoxidation of many polyhydric phenols, hydrogen peroxide is formed, many of the earlier investigators were inclined to the view that hydrogen peroxide may also be formed in the enzymic oxidation of catechol (2, 3, 4). On the other hand, Pugh and Raper (5), reported that two atoms of oxygen are taken up when catechol is oxidized by tyrosinase preparations which also contained catalase. If hydrogen peroxide is one of the oxidation products, the second atom of oxygen should be returned as the catalase decomposed the hydrogen peroxide. Likewise, Dawson and Ludwig (6), after an exhaustive study as to whether hydrogen peroxide occurs as one of the oxidation products when catechol is oxidized by tyrosinase, reached the conclusion that no evidence could be found supporting the view that it is formed.

Pugh and Raper (5) have suggested that the second atom of oxygen is involved in a further oxidation of the *o*-quinone, formed by means of the first atom of oxygen, to a higher oxidation product, the chemical nature of which is still undetermined. Since then, Wagreich and Nelson (7) have succeeded in confirming experimentally this suggestion of Pugh and Raper and also have shown that this higher oxidation product of the *o*-quinone is hydroxyquinone, and that the enzymic oxidation of catechol takes place in two steps.

o-Benzoquinone is the first oxidation product and involves the consumption of one atom of oxygen. It is well known that *o*-benzoquinone is very unstable in aqueous solution, and by following this disappearance of the quinone, they found that not only was the rate of disappearance monomolecular, but that half of the quinone is returned to the solution as catechol.
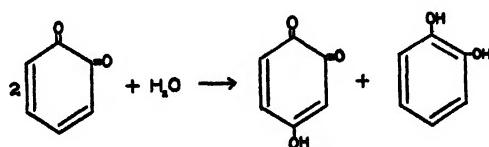
In the light of this information it becomes evident that when tyrosinase is present the newly formed catechol will be oxidized to *o*-benzoquinone. In other words, the final product in the enzymic oxidation of catechol is hydroxyquinone, and the role played by the tyrosinase is solely the oxidation of catechol to *o*-benzoquinone.

Two difficulties are encountered in attempting to use catechol as the substrate in studying the kinetics of the reaction. In the first place, the reaction is so fast that it is almost impossible to measure the rate with any degree of accuracy. If an attempt is made to cut down the rate by using a smaller amount of the enzyme, then it is found that the reaction soon comes to an end because of the inactivation of the tyrosinase.

This tendency of the tyrosinase to become inactivated when it catalyzes the oxidation of catechol has been noted by several investigators. Most of them (8, 9) have been inclined to attribute this inactivation of the enzyme to a denaturation of the enzyme protein by the quinone formed in the reaction.

In the light of experience from the study of tyrosinase, Ludwig and Nelson (10) have been led to question this explanation for the inactivation of the tyrosinase, and for this reason have studied this phenomenon in more detail.

When catechol is oxidized by means of an excess of tyrosinase, two atoms of oxygen are consumed per mole of catechol, as has already been mentioned. If, however, less than a sufficient amount of the enzyme is used, then the enzyme becomes inactivated before all the catechol is completely oxidized (curves I and II, Fig. 1.). When the tyrosinase has been sufficiently purified, so as to remove any catechol or its derivatives which occur together with the enzyme in the plant, it was found that the amount of oxygen consumed in the inactivation of one unit of the enzyme (ca-
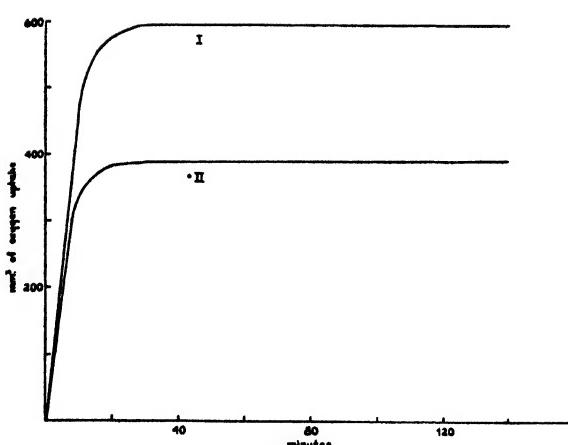
Fig. 1. Showing the inactivation of tyrosinase (*Psalliota campestris*) by an excess of catechol.

techolase) was 100 mm.³ In fact this amount of oxygen is so constant that it has proved to be a useful means of estimating the amount of tyrosinase present in a given preparation. It should be pointed out, however, that this 100 mm.³ of oxygen necessary for the inactivation of 1 unit of enzyme is true only for tyrosinase prepared from the common mushroom, *Psalliota campestris.* Tyrosinases from other sources require different amounts of oxygen for the inactivation of one unit. Thus tyrosinase from the wild mushroom, *Lactarius piperatus,* requires 400 mm.³, while the catecholase from the sweet potato requires only about 20 mm.³

There are several reasons for excluding the claim that the inactivation is due to the quinone formed when the catechol is oxidized. Thus removing the quinone as soon as it is formed, by the presence of sodium benzene sulfinate (11), does not change the amount of oxygen uptake necessary for the inactivation. Exposing the enzyme for some time to the quinone formed in the reaction has no effect, since the amount of oxygen uptake necessary for the inactivation of 1 unit was still 100 mm.³ The time required for the uptake of a given amount of oxygen or the oxidation of a given amount of catechol can be lengthened in different ways. For example, the reaction can be carried out at a lower oxygen concentration, say 2 p.c. oxygen instead of 21 p.c. as in air. Fig. 2 shows the rate of oxidation of catechol (excess) by a given amount of tyrosinase in the presence of air and in the presence of 2 p.c. oxygen and 98 p.c. nitrogen. It will be observed that the amount of oxygen uptake for the inactivation of the enzyme was the same in both instances. Another method for slowing up the reaction so as to expose the enzyme for a longer time to the reaction products before it is inactivated is to cut

down the concentration of the catechol. This can be done by starting with a low concentration of the catechol and having present in the reaction mixture an excess of a reducing agent such as ascorbic acid. The latter will reduce the *o*-benzoquinone, as soon as it is formed, back to catechol and thereby keep the concentration of the catechol constant. In this way it was also found that 100 mm.³ of oxygen was required to inactivate 1 unit of the tyrosinase from *Psalliota campestris.*

This inactivation of tyrosinase is independent of the hydrogen-ion concentration. In the table below are given the amounts of oxygen uptake for the inactivation of 1 unit of the enzyme at various values of pH.

pH	mm.³ of O_2 per unit
4.0	67
4.3	86
4.8	90
5.2	95
5.8	95
6.2	95
6.6	95
7.6	93
8.1	82
8.6	78

The low oxygen uptakes occurring below pH 5 are due to the instability of tyrosinase in solutions of this degree of acidity.

Temperature, however, does affect the inactivation. Thus at 25° C. the oxygen uptake is 100 mm.³ while at 35° C. only 80 mm.³ is required for bringing about the inactivation of 1 unit.

It has been mentioned already that it is possible to carry on the oxidation of catechol when the
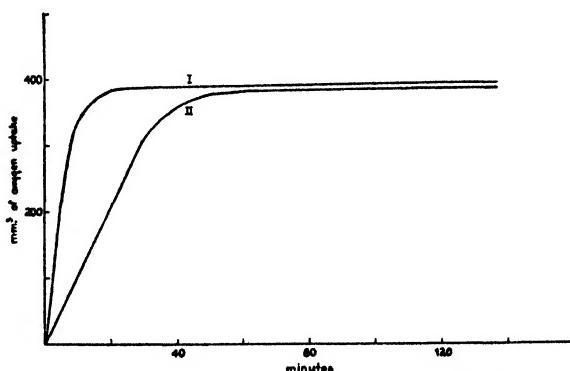
Fig. 2. Showing the inactivation of 4 units of tyrosinase (*Psalliota campestris*) in the presence of an excess of catechol. Curve I in the presence of air (21 p.c. oxygen). Curve II in the presence of 2 p.c. oxygen and 98 p.c. nitrogen.

latter is present in very low concentrations, provided some reducing agent, such as ascorbic acid, is present to change the quinone back to catechol. This is a convenient method in some kinetic studies of tyrosinase action in which it is desirable to hold the concentration of the substrate constant. When hydroquinone is the reducing agent this, however, is not the case. Besides reducing the o-quinone, it also exerts a protective influence on the enzyme so that many times more than 100 mm.[3] of oxygen is taken up per unit before the enzyme becomes inactivated. This can be seen on comparing curves I and III in Fig. 3. p-Cresol also exerts a protective influence on the tyrosinase in much the same way as the hydroquinone does (curve IV, Fig. 3). Other substances, such as benzoic acid, only act as retardants, but have no protecting influence. This will be seen on comparing curves I and V, Fig. 3.

Turning to the action of tyrosinase on p-cresol one of the striking differences is the very rapid oxidation of the catechol (curve I. Fig. 4) compared to the initial lag period followed by a practically constant rate of oxidation in the case of p-cresol (curve II, Fig. 4). Furthermore, the rates of oxidation for the two substrates bear no relation to one another. Different preparations from the same source, say *Psalliota campestris,* often vary over 20 fold in respect to the ratio between their catalytic influence on the rates of oxidation of the two substrates. Tyrosinase preparations from other sources vary even more. For this reason it has been necessary in studying the quantitative aspects of tyrosinase action to treat tyrosinase as two distinct enzyme actions. When p-cresol was the substrate the enzyme has been termed "cresolase" and when catechol has been used as the substrate, "catecholase". The unit of catecholase activity used in this paper is that described by Adams and Nelson (12) and the units of cresolase activity is that of Graubard and Nelson as modified by Adams and Nelson.

Fig. 4. Showing the relative rates of oxidation of p-cresol and catechol by a tyrosinase preparation (from *Psalliota campestris*) containing 5 units of catecholase and 2 units of cresolase. Curve I 2 mg. catechol. Curve II 2 mg. of p-cresol.

It has been known for some time that the addition of a trace of catechol to the reaction mixtures of p-cresol and tyrosinase immediately removes this initial lag period and the oxidation proceeds at a constant rate until most of the cresol has been oxidized. Considerable speculation has arisen as to just how the catechol overcomes this initial lag in the oxidation of the cresol. One of the theories most generally accepted is that of Onslow and Robinson (13). According to this view the monohydric phenol is oxidized spontaneously to the o-dihydric condition by the o-quinone or hydrogen peroxide formed in the oxidation of the added catechol.
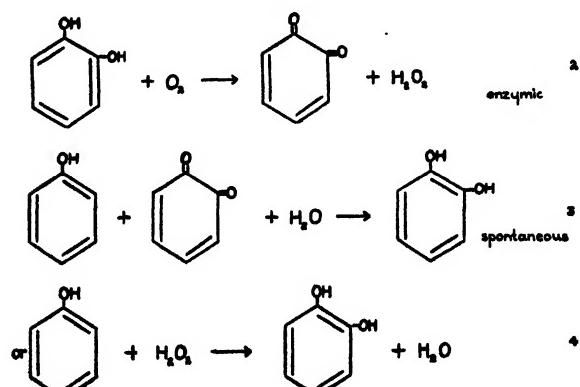
Recently Califano and Kertesz (14) claimed to have confirmed reaction 3 experimentally. Bordner and Nelson (11), however, have been able to show that phenol (or cresol) is not oxidized spontaneously by o-benzoquinone. By following the rate of disappearance of o-benzoquinone from an aqueous solution iodometrically they found that

Fig. 3. Showing the influence of hydroquinone, ascorbic acid, p-cresol and benzoic acid on the inactivation of tyrosinase (*Psalliota campestris*) by catechol.

the presence of phenol or *p*-cresol in the absence and in the presence of tyrosinase had no effect. By a similar procedure they were able to exclude reaction 4, *i.e.* that hydrogen peroxide oxidizes the phenol to the *o*-dihydric condition. They were also able to exclude the suggestion made by Richter (3) that reaction 3 above is brought about by the presence in the tyrosinase preparations of a monohydric phenol dehydrogenase, which uses *o*-quinone as a hydrogen acceptor.

Since the shortening of the initial lag period in the oxidation of monohydric phenols cannot be due to the action of either *o*-quinone or hydrogen peroxide, it became necessary to look for some other explanation. Tyrosinase seems to be unable to act directly upon monohydric phenols, unless it is first permitted to take part in the oxidation of an *o*-dihydric phenol. In other words, a trace of the *o*-dihydric compound is necessary for initiating the oxidation of the monohydric phenol.
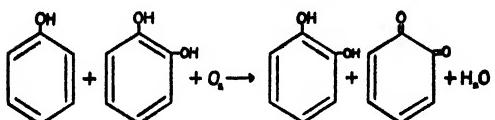
Furthermore, in order for the reaction to be autocatalytic the amount of the *o*-dihydric phenol present must gradually increase. According to the observations of Wagreich and Nelson, already mentioned, this gradual increase of the *o*-dihydric phenol is just what would be expected. It will be recalled that they showed that when *o*-benzoquinone disappears in an aqueous solution, half of the quinone is changed back into catechol.
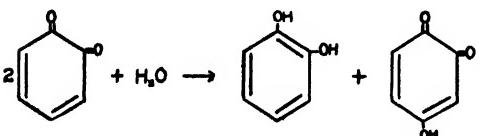
Considering reactions 5 and 6 together, it becomes evident that an ever-increasing amount of *o*-dihydric phenol is oxidized per unit time, thus enabling the tyrosinase to act upon an ever increasing amount of the monohydric phenol. As the rate of oxidation of the monohydric phenol increases autocatalytically, a maximum rate is finally reached when the enzyme is working at full capacity, after which the rate remains practically constant for a considerable length of time. According to the above view, the mechanism involved in the oxidation of monohydric phenols is the oxidation of catechol which so modifies the tyrosinase that it can bring about the oxidation of the monohydric phenol. Any condition at the

beginning of the reaction which would tend to decrease the concentration of catechol should lengthen the initial lag period, and likewise any condition which should tend to increase the amount of catechol should tend to decrease the duration of the lag period. This has been found to be the case. For example, the addition of oxidizing agents, such as potassium ferricyanide, or a suspension of manganese dioxide, would oxidize some of the catechol leaving less to activate the tyrosinase towards monohydric phenol, and thus lengthen the lag period (curve III, Fig. 5). Like-
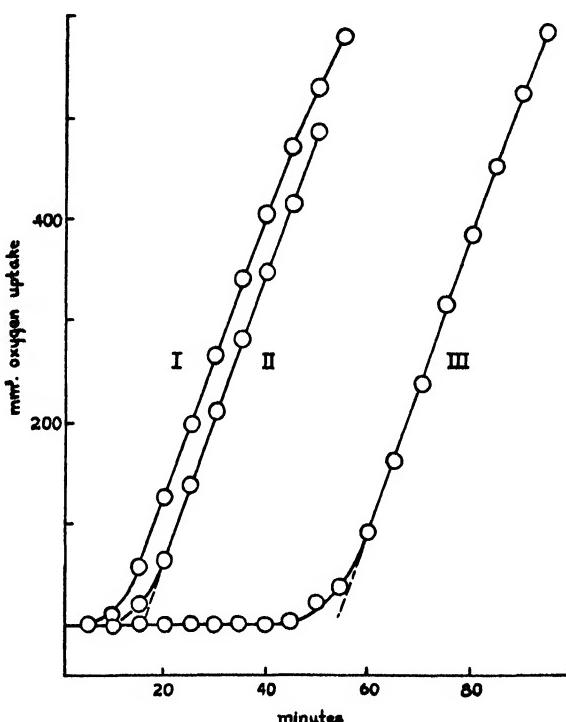
Fig. 5. Curve II (Control) *p*-Cresol oxidized by tyrosinase at pH 6. Curve III. Oxidation of *p*-cresol by tyrosinase in the presence of $K_3Fe(CN)_6$ at pH 4. Curve I. Oxidation of *p*-cresol by tyrosinase at pH 7.

wise the presence of borates, which are known to combine with catechol, has a similar influence.

Conditions such as higher hydroxyl-ion concentration, which Dawson and Nelson (15) have shown increases the rate of reaction 6 and thereby increases the amount of catechol, have been found to shorten the initial lag period (curve I, Fig. 5). On the other hand, the addition of sodium benzenesulfinate which combines with *o*-benzoquinone tends to decrease the formation of catechol and thus lengthens the initial lag period. Reducing agents such as potassium ferrocyanide, alanine and hydroquinone, which tend to reduce the *o*-benzoquinone and increase the amount of catechol, were found to shorten the lag period.

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Discussion

Dr. Bernheim: Have you any idea as to what group in the tyrosinase molecule is inactivated? Might it possibly be an SH group, since some of the reagents you use are known to oxidize and reduce SH groups in protein?

Dr. Nelson: So far we have no definite information concerning the nature of the inactivation.

Dr. Goddard: If I remember correctly Keilin's preparations of polyphenol oxidase from mushrooms had no action on tyrosine. Do you consider tyrosinase and catechol oxidase as identical?

Dr. Nelson: Tyrosinase prepared from the common mushroom, *Psalliota campestris,* catalyzes the oxidation of tyrosine, but 4 to 5 times more slowly than *p*-cresol under similar conditions. The term tyrosinase implies that the enzyme, in the presence of a trace of catechol, catalyzes the aerobic oxidation of certain monohydric phenols. Catecholase, or catechol oxidase, is defined as the enzyme which catalyzes the oxidation of certain *ortho*-dihydric phenols. Keilin and Kubowitz and some other workers regard tyrosinase as a polyphenolase, *i.e.* an enzyme which oxidizes polyhydric phenols. This view, according to our experience, is too broad. Phenolases from certain sources, as for example *Russula foetens,* bring about the aerobic oxidation of hydroquinone and catechol, while the phenolases from the common mushroom and from the sweet potato have practically no action on hydroquinone.

I might also add that we have not found any enzyme, capable of oxidizing monohydric phenols, which does not also oxidize catechol. On the other hand, there are catecholases which do not oxidize monohydric phenols.

Dr. Goddard: In these experiments are tyrosine and *p*-cresol interchangeable?

Dr. Nelson: They are interchangeable as far as the aerobic oxidation of both being catalyzed by tyrosinase. But, as has already been pointed out, *p*-cresol is oxidized at a faster rate than the tyrosine.

Dr. Goddard: Is one safe in using *p*-cresol instead of tyrosine?

Dr. Nelson: It all depends on what your problem is. If you are interested, from the standpoint of genetics, in pigment formation, probably you would use tyrosine or tyramine. On the other hand, if you are interested in gaining a better understanding of the reactions involved in the introduction of the second hydroxyl group, *ortho* to the hydroxyl already present in a monohydric phenol, then you would very likely use *p*-cresol.

Dr. Graubard: One should use *p*-cresol rather than tyrosine, because there are complications involved in connection with tyrosine. You very often have a good tyrosinase preparation that will work on *p*-cresol with some lag but will not work on tyrosine.

There is one difficulty in connection with the sweet potato oxidase. You will remember that at the beginning when you get the sweet potato oxidase out it occasionally reacts with *p*-cresol.

Dr. Nelson: The oxidase from sweet potato has practically no action on *p*-cresol.

Dr. Graubard: If you take the sweet potato oxidase and add catechol to it, even though you prime it, it will not do anything with *p*-cresol. How do you explain that?

Dr. Nelson: So far we have not had the time to go into that question.

Dr. Barker: I believe that you explained reactions as starting off with some action of the catechol on the enzyme. Do you have any idea as to what actually takes place, and what would be the intermediate steps?

Dr. Nelson: All we know at the present is that the enzymic oxidation of a monohydric phenol, such as *p*-cresol, starts out with a lag period in which practically no oxygen uptake occurs. The addition of a trace of catechol removes this lag period. Either something is formed in the enzymatic oxidation of the catechol which is necessary for the oxidation of the *p*-cresol, or the enzyme is modified in some way, while oxidizing the catechol, and is thereby enabled to catalyze the oxidation of the monohydric phenol.

Dr. Salomon: I understand that you have some indication that tyrosinase is a copper-containing enzyme. Would you tell us more about this point?

Dr. Nelson: Just as Keilin and Mann and Ku-

bowitz have obtained enzyme preparations which catalyze the aerobic oxidation of *p*-cresol and catechol and have shown them to be copper proteins, so have we. The activity of our preparations towards catechol parallels the copper content, just as they report.

Furthermore we have succeeded in obtaining a crystalline product from very concentrated solutions of a tyrosinase, prepared from the wild mushroom, *Lactarius piperatus*. These crystals have the same composition, 0.25 p.c. copper and 13.6 p.c. nitrogen, as the highly purified tyrosinase from the same source. The crystalline material was only slightly active, and we are not sure whether this slight activity belonged to the crystals or whether we didn't succeed in removing completely all of the very active mother liquor.

Dr. Salomon: Last year we carried out experiments concerning the catalytic activity of polyphenol oxidase and that of the copper-protein compound present in serum. We compared the catalytic power of the two substances in the system polyphenol oxidase-pyrocatechol-ascorbic acid and in the copper-protein compound of serum pyrocatechol-ascorbic acid. When human or pig serum was added instead of potato oxidase no catalytic action could be observed.

Dr. Barker: Once you have gone out on the plateau of inactivation of the enzyme, is there any possibility of reactivation?

Dr. Nelson: No. We have tried in several ways to reactivate the catecholase, after it has been inactivated by acting on an excess of catechol, but so far we have not met with any success.

Dr. Goddard: Have you tried to protect the enzyme from inactivation by *p*-phenylene diamine?

Dr. Nelson: No. We have only used hydroquinone.

Dr. Goddard: In intact plants, if catechol is added, there is often a very rapid inactivation of the enzyme, but the enzyme retains its activity if *p*-phenylene diamine is added with the catechol,

even when the enzyme has no primary action on *p*-phenylene diamine. Hydroquinone and *p*-phenylene diamine might almost be interchangeable in the system.

Dr. Graubard: But they really are not. If you use a little bit of catechol in five milligrams of hydroquinone, you can oxidase the hydroquinone, but if you do that with *p*-phenylene diamine, nothing happens. The latter apparently combines with the catechol in the way aniline does.

Dr. Goddard: Then *p*-phenylene diamine is precipitated out of the system.

Dr. Nelson: Possibly some remarks would not be out of place concerning the phenolase which oxidizes hydroquinone directly without the intervention of catechol. It often has been called "laccase" although it is different from the laccase obtained from the lactree. A good source for this phenolase is the wild mushroom, *Russula foetens*. It oxidizes hydroquinone quite rapidly, while in the case of catechol the rate of oxygen uptake is much slower. If, however, a small amount of catechol is added to the reaction mixture when hydroquinone is serving as the substrate, then the rate of the reaction is lowered a great deal. It seems as though a distribution of the enzyme occurs between the two substrates. Even though the enzyme catalyzes the oxidation of the hydroquinone at a faster rate than it does the oxidation of the catechol, still the latter has a greater affinity than hydroquinone for the enzyme.

This same indication of a distribution of the enzyme between different substrates also shows up in the case of tyrosinase. Catechol is oxidized very rapidly by the enzyme (Curve I in Fig. 4). *p*-Cresol is, under the same conditions, oxidized at a slower rate (Curve II, Fig. 4). Yet if catechol is added to the reaction mixture containing *p*-cresol as the substrate, aside from removing the induction period, it has practically no influence on the rate of oxygen uptake. The rate is still the same as that shown by Curve II.

THE ROLE OF IRON-PORPHYRIN COMPOUNDS IN BIOLOGICAL OXIDATIONS

E. S. Guzman Barron

The iron-porphyrin compounds occupy a unique position among the oxidation-reduction systems of biological importance, because on combining with nitrogenous compounds they form complexes possessing manifold properties, all of them connected with the function of respiration. Thus, certain iron-prophyrins may combine with a protein and form compounds—hemoglobin, myoglobin, chlorocruorin, erythrocruorin—which have the property of combining reversibly with molecular oxygen with no alteration of the valence number of the Fe atom. Iron-porphyrins may combine with proteins and give non-autoxidizable sluggish oxidation-reduction systems—hemoglobin-methemoglobin, cytochrome-c, cytochrome-a. Finally, they may combine with proteins or other nitrogenous compounds giving autoxidizable, electroactive systems—hemochromogens, cytochromes-b and -a₃.

All the iron-porphyrin compounds which take part in cellular respiration seem to have derived from etioporphyrin III (1, 3, 5, 8-tetramethyl, 2, 4, 6, 7-tetraethylporphyn). Hemoglobin, cytochrome-c, catalase and possibly peroxidase and cytochrome-b have protoporphyrin IX as the porphyrin nucleus. Cytochromes-a and -a₃ and Warburg's *"Sauerstoffübertragendes Ferment"* seem to have a porphyrin similar to that of *Spirographis* porphyrin, the constitution of which was elucidated by Fischer and Seeman (19). In *Spirographis* porphyrin the vinyl group of protoporphyrin in 2 position is replaced by a formyl group.

Oxygen transport iron-porphyrin compounds. The iron-porphyrin compounds of this group by coupling with globin have acquired the unique property of being able to combine reversibly with molecular oxygen and thus act as oxygen stores in highly developed multicellular organisms. These oxygen stores possess varied affinities for molecular oxygen and varied rates of combination, a property which allows the quick loading and unloading of molecular oxygen from the air to the tissues. The half oxygen dissociation of horse myoglobin at pH 7.46 occurs at 3.26 mm. O_2 pressure (Theorell, 1). It has six times as great an affinity for oxygen as horse hemoglobin has under identical conditions.

In 1930, Barron and Hoffman (10) found that the catalytic power of reversible dyes on cellular respiration was conditioned by two factors, the oxidation-reduction potential of the dye and the permeability of the cell membrane. A study of the rate of oxidation by atmospheric oxygen of reduced electroactive dyes showed the same relation between the free energy and the rate of oxidation (Barron, 4). A similar relation has been found by Millikan (39) in these oxygen store systems. The rate of combination of oxygen with myoglobin, the system with greater affinity for oxygen, is 2.5 to 5 times as fast as that of hemoglobin for oxygen. There is a relation between equilibrium constants and rates of reaction. The role of myoglobin as an oxygen store has been demonstrated by Millikan (40) in experiments *in vivo* by measuring the instantaneous oxygen consumption of the soleus muscle of the cat. The onset of oxygen consumption started at the same time as the onset of contraction. According to him, the special provision for oxygen storage in the form of myoglobin suggests that this quick aerobic mechanism is the efficient and satisfactory one. This opinion finds support in Hurtado's (24) important observation of the relative increase of blood and muscle hemoglobin of dogs when brought up to high altitudes. While there was a gain of about 44 p.c. in hemoglobin, there was a gain of 66 p.c. to 70 p.c. in myoglobin. As the oxygen transporting power of hemoglobin diminished because of the low oxygen tension in high altitudes, the concentration of the tissue oxygen transport system, myoglobin, increased.

Sluggish oxidation-reduction systems. The iron-porphyrin compounds belonging to this group may be reversibly oxidized and reduced with the use of suitable agents, the oxidation and reduction being performed by the loss or addition of one electron to the iron atom of the porphyrin. These compounds are not electroactive, *i.e.*, their potentials cannot be promptly determined with the use of indifferent electrodes and the usual potentiometric technique unless electroactive mediators are added to the system. They are not autoxidizable, *i.e.*, not easily oxidized by atmospheric oxygen.

Hemoglobin and myoglobin may act as sluggish oxidation-reduction systems, as shown by Conant (14) and Taylor (50). As such, the system hemoglobin-methemoglobin no longer acts as an oxygen transporter, but as an oxidizing agent, its function being limited by the slow rate of oxidation of hemoglobin to methemoglobin (Neill and Hastings, 41). Warburg, Kubowitz, and Christian (58), on studying the catalytic action of methylene blue on the respiration of red cells found by Harrop and Barron (23), postulated that in the presence of the dye hemoglobin was oxidized to methemoglobin, which in turn oxidized lactic acid to pyruvic acid:

I.   M.B. (methylene blue) $+$
Hb(hemoglobin) $+$ H_2O $\rightarrow$
L.M.B.(leucomethylene blue) $+$
MetHb(methemoglobin)

II.   Lactic acid $+$ MetHb $\rightarrow$ pyruvic acid $+$ Hb

III.   L.M.B. $+$ O_2 $\rightarrow$ M.B. $+$ H_2O_2

The power of methemoglobin to raise the oxygen consumption of red cells was demonstrated by them in experiments where the dye was replaced by methemoglobin, the increased oxygen consumption stopping in such cases as soon as the methemoglobin was reduced. DeMeio, Kissin and Barron (17), in experiments with dyes of a potential sufficiently negative to avoid oxidation of hemoglobin, showed that the catalytic effect of dyes was independent of methemoglobin formation. Undoubtedly, if dyes able to oxidize hemoglobin are used, Warburg's scheme may be partly or wholly responsible for the increased oxygen consumption. It seems improbable that either methemoglobin or metmyoglobin acts as an oxidation catalyst in living cells, because of the slow rate of oxidation of the ferro-compound.

Keilin's cytochromes-a and -c are the most important components of this group. Cytochrome-a, according to Keilin, is an iron-porphyrin protein compound which does not combine with HCN and CO, and is not autoxidizable. We know almost nothing about its chemical constitution, since it has not yet been isolated. It is possible that the porphyrin group of cytochrome-a is similar to that of *Spirographis* hemin (Roche and Benevent, 44). The oxidation-reduction potential of this cytochrome, according to Ball (3), is about +0.29 volt.

With the establishment of simple methods for the preparation of cytochrome-c (Keilin and Hartree, 27; Theorell, 52), it has become possible to determine its chemical constitution. The porphyrin group of cytochrome-c, according to Theorell's important contribution (54), is 1, 3, 5, 8-tetramethyl, 2, 4-diethyl, 6, 7-dipropionic acid porphyn, *i.e.*, identical to the porphyrin group of hemoglobin. Two S atoms appear to be present in the form of thio-ether linkages forming a bridge from the porphyrin to the protein. On one side, the S atoms are linked to the 2 and 4 position of porphyrin; on the other side, the S atoms are attached to C atoms in the diamino-dicarboxylic acid side chain. One or several of these amino and carboxyl groups are linked by peptide bonds to the portion of the protein component which is split off by acid hydrolysis. The protein component is also bound to the Fe atom.

The oxidation-reduction potential of cytochrome-c has been redetermined by several workers (Ball, 3; Laki, 33; Stotz *et al.*, 47; Wurmser and Filitti-Wurmser, 60), the values obtained by them agreeing fairly well with those obtained previously by Coolidge (16). The E'_o of the system is +0.257 volt from pH 5 to 8; *i.e.*, the potential is, within these limits, independent of the hydrogen ion concentration, a property similar to that exhibited by cyanide hemochromogen. Cytochrome-c, which is not oxidized by atmospheric

oxygen at measurable rates between pH values of 4 and 12, becomes autoxidizable below pH 4 and above pH 12. In neutral solutions it is oxidized by cytochrome oxidase (Keilin and Hartree, 29).

Electroactive iron-porphyrin compounds. The third group of iron-porphyrin compounds comprises those systems which behave as thermodynamically reversible oxidation-reduction systems and which are electromotively active, *i.e.*, whose potentials are easily determined potentiometrically with indifferent electrodes, in the classical way. These systems are autoxidizable, *i.e.*, easily oxidized by atmospheric oxygen. As models of this group, the iron-porphyrin compounds from hemoglobin and chlorocruorin may be taken. Blood hemin (the FeCl compound of 1, 3, 5, 8-tetramethyl, 2, 4-divinyl, 6, 7-dipropionic acid porphyn) and *Spirographis* hemin (the FeCl compound of 1, 3, 5, 8-tetramethyl, 2-formyl, 4-vinyl, 6, 7-dipropionic acid porphyn) can be reduced by suitable agents and reoxidized with air. As Anson and Mirsky (2) discovered in 1925, they combine reversibly with nitrogenous compounds forming a coördination complex which we usually call hemochromogen. In its most simple form this may be expressed as follows:

$$Fe^{++} + 2N \text{ (nitrogenous compound)} \rightleftharpoons Fe^{++}(N)_2$$

$$Fe^{+++} + 2N \rightleftharpoons Fe^{+++}(N)_2$$
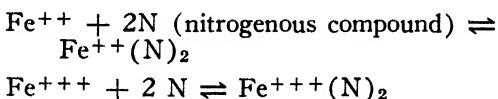
These nitrogenous compounds are formed as soon as they come in contact with the iron-porphyrin and with such ease that one hemochromogen can be changed into another according to the value of their dissociation constants. Moreover, the temperature coefficients of some of these compounds are so high that they may be dissociated on changing the temperature of the solution. The importance of these properties cannot be overemphasized, because of their possible application to naturally occurring hemochromogens, and because of the difficulties they may cause to workers interested in the isolation of these natural hemochromogens. Keilin, in his extensive work on cytochromes, has reported examples of these difficulties, and in a recent paper Keilin and Hartree (30), say, speaking of cytochrome-a: "On denaturation the *a* band of these compounds moves towards the blue end of the spectrum to occupy a position at about 583 mμ. This derivative (of -a and -a$_3$) has the properties of an ordinary hemochromogen, namely, it is autoxidizable and combines with CO."

Conant and his collaborators (15) were the first to demonstrate that both the ferrohemin-ferrihemin system and the ferrihemochromogen-ferrohemochromogen system are electromotively ac-

tive reversible oxidation-reduction systems, a one electron transfer being involved in the process. For blood hemin (Barron, 5) the potential of the system at 30° C. is obtained from the equation:

$$E_h = E_o - 0.06 \log \frac{[Fe^{++} \text{ hemin}]}{[Fe^{+++} \text{hemin}]}$$

$$- 0.06 \log \frac{1}{[H^+]}$$

where E_o (the normal potential of the system at pH 0) in phosphate buffer was calculated to be +0.306 volt, and in borate buffer, +0.263 volt. The potentials of blood hemin became more positive on addition of a nitrogenous compound, the rise in potential being in inverse relation to the affinity of the nitrogenous compound for hemin. It was also found that when the affinity of the nitrogenous compound for hemin was great, as in cyanide hemochromogen, the potential was independent of the hydrogen ion concentration; when the affinity was low, as in nicotine, α picoline, and pyridine hemochromogens, the E_h values increased, as in hemin, by 60 millivolts per pH unit. The values of the potentials of *Spirographis* hemin and some of its hemochromogens (Barron, 6) are more positive than those of blood hemin and the corresponding hemochromogens, a finding which is of importance because, as already stated, Warburg's *"Sauerstoffübertragendes Ferment"* and Keilin's cytochromes-a and -a₃ seem to have the porphyrin nucleus similar to that of *Spirographis* porphyrin. The iron-porphyrins combine reversibly with the nitrogenous substances, the metal being attached by covalent bonds not only to the porphyrin nitrogens but also to the nitrogen atoms of the nitrogenous substances (Pauling and Coryel, 42). Clark and his co-workers (12) have given a clear quantitative treatment of these empirical findings. By introducing the dissociation constants of the hemochromogens into the electrode equation, they have postulated that if the affinity of the nitrogenous compound for ferri- and ferrohemochromogen is the same, there will be no change of potential on addition of the nitrogenous base. If the potential becomes more positive it means that the reductant forms the more stable compound; if the oxidant associates more strongly than the reductant the trend of the potential will be in the negative direction. Table I gives the potentials (E_o values) at pH 7.0 of cytochromes-a, -b, and -c, and of the iron porphyrins and hemochromogens. One active nucleus, iron-porphyrin, because it combines reversibly with a number of nitrogenous substances with different degrees of affinity, can alter its free energy so widely as to

Table I

Oxidation-reduction potentials of hemins and hemochromogens

E'_o values at pH 7.0, temp. 30° C.

System	E'_o
	volts
Blood hemin (phosphate)	—0.110
Nicotine hemochromogen	+0.184
Pyridine hemochromogen	+0.172
α-Picoline hemochromogen	+0.136
Histidine hemochromogen	—0.106
Globin hemochromogen	—0.098
Cyanide hemochromogen (pH 8.2)*	—0.183
Spirographis hemin	—0.077
α-Picoline *Spirographis* hemochromogen	+0.155
Pilocarpine *Spirographis* hemochromogen	—0.024
Cyanide *Spirographis* hemochromogen (pH 8.2)*	—0.113
Cytochrome-a, pH 7.4	+0.29
Cytochrome-b, pH 7.4	—0.04
Cytochrome-c, pH 7.4* . . .	+0.255

* Potentials independent of pH.

give the clearest example of a series of oxidation-reduction systems of graded potentials.

Cytochrome-b, according to Keilin and Hartree (30), is autoxidizable, *i.e.*, oxidized by atmospheric oxygen. They state further that this oxidation may occur even in the presence of great excess of HCN, an observation which, if confirmed, would indicate that cytochrome-b may be able to act as an oxidation catalyst in the presence of HCN. The chemical constitution of this cytochrome is unknown. The E'_o value at pH 7.4, as given by Ball, is —0.04 volt. Keilin and Hartree have discovered lately (30) another autoxidizable cytochrome seemingly of more positive potential than cytochrome-a. This cytochrome (-a₃) is not only autoxidizable but combines reversibly with HCN and CO, the position of the absorption band of the CO compound being similar to that assigned by Warburg and his co-workers (55) to the CO compound of his *"Sauerstoffübertragendes Ferment"*. They suggest that this cytochrome may be the cytochrome oxidase.

The role of iron-porphyrins in biological oxidations. The first group of iron-porphyrin compounds, those which combine reversibly with molecular oxygen, have a function of first importance in multicellular animal organisms, as they transport the oxygen from the air to the cells for the performance of the oxidation processes. These compounds are not catalysts for oxidation proc-
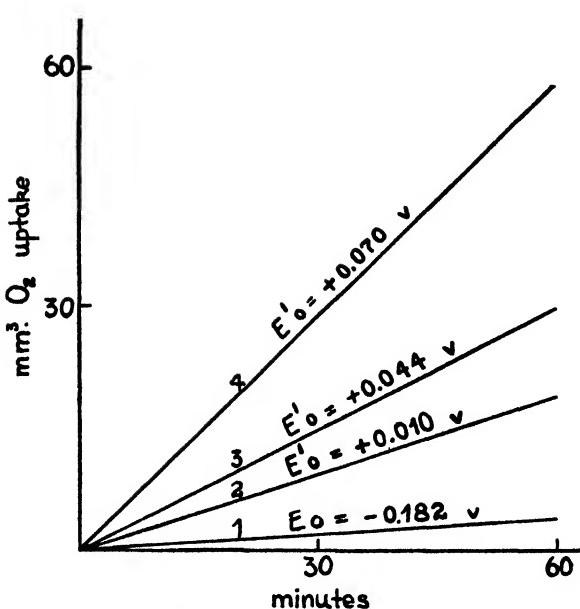
Fig. 1. The effect of hemochromogens on the oxidation of lactate activated by α-hydroxyoxidase. pH 9.0. Temperature, 37° C. Curve 1, cyanide hemochromogen; curve 2, picoline hemochromogen; curve 3, pyridine hemochromogen; curve 4, nicotine hemochromogen. (Unpublished data.)

esses. The function of the second group of iron-porphyrin compounds, *i.e.*, the sluggish, non-autoxidizable oxidation-reduction systems, cytochromes-c and -a, is not yet clear. If they act as other sluggish systems, pyridine nucleotides for example, they will function as electron mediators in the series of oxidation-reduction systems, placed between oxidizable substrate and molecular oxygen. As dihydropyridine nucleotides are reoxidized through the mediation of alloxazin dinucleotide compounds (Euler's diaphorase (1) or Green's cozymase factor (18)) so these cytochromes are reoxidized through the mediation of cytochrome oxidase.

The electromotively active iron-porphyrin compounds seem to act by virtue of their oxidation by molecular oxygen, as the last members of the series of oxidation systems, transferring electrons to molecular oxygen. Let us take the oxidation of lactic acid by α-hydroxyoxidase prepared from gonococci. This oxidation differs from the oxidation of lactic acid in muscle tissues in that it does not require the presence of diphosphopyridine nucleotide. It requires a protein to activate the oxidizable molecule and the cytochrome system to perform the oxidation. By heating the preparation for one hour at 60° C. cytochrome oxidase is destroyed and lactic acid is no longer oxidized. The oxidation starts as soon as hemin or hemochromogens are added to the suspension (Barron

and Hastings, 9; Barron, 7). The rate of oxidation of lactic acid increases as the potentials of the iron-porphyrin compounds added become more positive (Fig. 1).

Hemochromogens made in the laboratory may be taken as models for the understanding of the function of natural hemochromogens. Barron *et al.* (8) and Lyman and Barron (37) studied the oxidation of sluggish oxidation-reduction systems, like ascorbic acid and glutathione. The oxidation is performed by the usual electron transfer from the oxidizable substrate to ferrihemochromogen and from ferrohemochromogen to oxygen:

1. Ascorbic acid $+ 2\,Fe^{+++} \rightarrow$ dehydroascorbic acid $+ 2\,Fe^{++} + 2\,H^+$

2. $2\,Fe^{++} + O_2 \rightarrow 2\,Fe^{+++} + H_2O_2$

The H_2O_2 formed in this reaction oxidizes the hemochromogen further, the porphyrin ring being opened to give a bile pigment with the iron still attached (Lemberg, 34). Here, too, there is a relation between the catalytic power of the hemochromogen and its oxidation-reduction potential (Fig. 2). The ease with which these hemochromogens oxidize sluggish systems must be taken into consideration in work concerned with the isolation of cytochrome oxidase. Since blood hemin
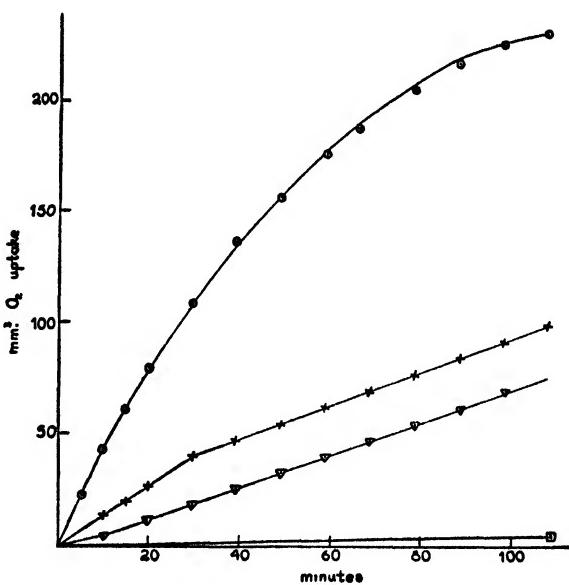
Fig. 2. The oxidation of ascorbic acid by atmospheric oxygen with hemochromogens as catalysts. pH 6.39. Temperature, 25° C. Amount of ascorbic acid, 0.02 mM. Amount of hemin in the hemochromogens, 0.00015 mM Curve 1, control; curve 2, pilocarpine hemochromogen; curve 3, pyridine hemochromogen; curve 4, nicotine hemochromogen. Curves numbered from bottom (Curve 1) to top (Curve 4).

with a potential 50 millivolts more negative than that of *Spirographis* hemin can form hemochromogens which will oxidize reduced cytochrome-c (nicotinic acid amide hemochromogen oxidizes reduced cytochrome-c), it is conceivable that spirographis hemin may form artificial hemochromogens able to oxidize reduced cytochrome-c.

When thiamin or diphosphothiamin is reduced with $Na_2S_2O_4$, the reduced compound is split into reduced thyazol and pyrimidine (Lipmann and Perlmann, 36). The reduced compound is not autoxidizable. Hemin and hemochromogens (histidine hemochromogen, for example) act as catalysts for the oxidation of reduced thyazol, the

Fig. 3. The oxidation of reduced thiamin by atmospheric oxygen with hemin and hemochromogens as catalysts. pH 7.26. Temperature, 38°C. Curve 1, reduction of thiamin by $Na_2S_2O_4$ as measured by CO_2 output in $NaHCO_3$ buffer with N_2: CO_2 as gas phase; curve 2, oxidation of reduced thiamin with histidine hemochromogen (O_2 uptake); curve 3, oxidation with hemin (O_2 uptake). Curves numbered from top (Curve 1) to bottom (Curve 3). (Unpublished data.)

mechanism of the oxidation being similar to that of the oxidation of sluggish systems (Fig. 3). The oxidation may also be performed with cytochrome-c and Keilin's cytochrome oxidase suspension. Lipmann (35), in experiments with bacterial suspensions prepared from *B. Delbrückii*, found that in such suspensions pyruvic acid was oxidized on addition of diphosphothiamin and Warburg's alloxazin dinucleotide (the alloxazin component which acts as oxidizing catalyst for the oxidation of *d*-amino acids (Warburg and Christian, 56). Since the oxidation of pyruvic acid by animal tissues and bacteria is largely inhibited by HCN, it is our opinion that in the oxidation of pyruvic acid the cytochrome-cytochrome oxidase systems are the systems concerned with the transfer of electrons to molecular oxygen. If alloxazin dinucleotide is one of the components of a ketonoxidase it must act as intermediate electron-transferring catalyst.

All these oxidations where hemochromogens act as oxidation catalysts are inhibited by HCN and CO. Furthermore, the inhibition produced by CO is suppressed by light. Hemochromogens thus exhibit the fundamental properties attributed by Warburg to his *"Sauerstoffübertragendes Ferment"* and by Keilin to his cytochrome oxidase. It is therefore reasonable to assume that the iron-porphyrin compounds which act as the last members in the transfer of electrons from oxidizable substrate to molecular oxygen do so because they act as electroactive oxidation-reduction systems. These compounds therefore do not combine with molecular oxygen as hemoglobin does; they react with it, reducing oxygen to H_2O_2.

Iron-porphyrin compounds may act by initiating an oxidation which proceeds as a chain reaction. Such is the case in the catalytic action of hemin and hemochromogens on the oxidation of unsaturated fatty acids reinvestigated by Barron and Lyman (11). When either linseed oil or oleic acid was mixed with a ferrihemochromogen in the absence of oxygen, the spectrum of ferrohemochromogen appeared very slowly and the concentration of it never exceeded 8 to 10 p.c. Similar findings were made by Wright, Conant, and Kamerling (59) when studying the catalytic effect of ferricyanide in the oxidation of oleic acid. It seems likely that the function of iron-porphyrin compounds in cases similar to this is like that of Cu^{++} ions in the oxidation of sodium sulphite, which, according to Haber (22), is probably due to their causing an increase in the number of initial centers as compared with that in a pure solution. Once the chain reaction is started and the reaction velocity has reached its constant value, the inhibiting effect must be quite small compared with its action when added at the initial period. This is clearly shown in experiments with HCN

2. $4 \text{ Fe}^{++} + 4 \text{ H}^{+} + \text{O}_2 \rightarrow$
    $4 \text{ Fe}^{+++} + 2 \text{ H}_2\text{O}$

The ferri- form of catalase is reduced by H_2O_2 to the ferro- form, which is reoxidized by oxygen. The formation of the ferro- compound is postulated from experiments where the catalase-azide complex is made to react with H_2O_2. The greenish brown solution of azide-catalase turns red and the spectral bands characteristic of azide-catalase are replaced by two new bands which Keilin and Hartree consider to be the ferrous compound. The reoxidation of the Fe^{++} form by atmospheric oxygen, and not by H_2O_2, is postulated from experiments in the absence of oxygen, since, according to them, the decomposition of H_2O_2 is inhibited completely. These experiments need confirmation. As Stern (private communication) points out, it is difficult to accept without further experimentation the chemical passivity of the Fe^{++} compound towards such a strong oxidizing agent as H_2O_2 ($4 \text{ Fe}^{+++} + 2 \text{ H}_2\text{O}_2 + 4 \text{ H}^{+} \rightarrow 4 \text{ Fe}^{++} + 4 \text{ H}_2\text{O}$). This objection could be met by assuming that the Fe^{++} compound is oxidized by oxygen with greater speed than by H_2O_2. In

Fig. 4. Effect of HCN on the oxidation of linseed oil with cyanide hemochromogen as catalyst. pH 9.19. Temperature 38°C. Concentration of HCN, 0.0283 M. Concentration of cyanide hemochromogen, 8×10^{-5} M. Upper curve, HCN added 10 minutes after hemochromogen addition; lower curve, CHN added before hemochromogen addition.

as the catalyst inhibitor and cyanide hemochromogen as the catalyst of the chain reaction (Fig. 4). HCN added before addition of the catalyst produced a large and permanent inhibition (92 p.c.), but when it was added after the reaction velocity became constant, the inhibiting power was small indeed. On the other hand, a chain reaction inhibitor will inhibit the reaction velocity whether it is added at the start of the reaction or later, the inhibiting effect depending on the length of the chain and concentration of the inhibitor. p-Aminophenol, a chain reaction inhibitor, added both before and after addition of the catalyst inhibited the reaction to the same extent in both cases (Fig. 5).

Catalase, as shown by Stern (46), is a ferri-porphyrin-protein complex of a molecular weight of about 248,000 (Sumner and Gralen, 48). In contrast to the iron-porphyrin compounds passed in review, this ferri-porphyrin protein is not reduced by the usual reducing agents. Keilin and Hartree (28) have formulated the mode of action of catalase as follows:

1. $4 \text{ Fe}^{+++} + 2 \text{ H}_2\text{O}_2 \rightarrow$
    $4 \text{ Fe}^{++} + 4 \text{ H}^{+} + 2 \text{ O}_2$

Fig. 5. The effect of p-aminophenol (0.001 M) on the oxidation of linseed oil with hemin as catalyst. pH 6.96. Temperature, 28°C. Curve 1, control; curve 2, p-aminophenol added 20 minutes after hemin addition; curve 3, p-aminophenol added 10 minutes after hemin addition; curve 4, p-aminophenol added before hemin addition. Curves numbered from top (Curve 1) to bottom (Curve 4).

the absence of oxygen, the rate of reaction would be partly inhibited. Catalase may thus be called a hydrogen peroxide oxidase, its action being similar to that of the oxidizing action of hemochromogens.

Peroxidase is another iron-porphyrin-protein complex. Its mode of action is a further example of the multiple mechanisms by which iron-porphyrins function in respiration processes. The iron-porphyrin is, as in catalase, in the trivalent state. The ferrous form of the compound combines reversibly with CO. HCN forms a spectroscopically well defined complex with the ferric form. Its mode of action is entirely different from that of catalase. Keilin and Mann (31) have shown that peroxidase always remains in the ferric form. On the addition of H_2O_2 this iron-porphyrin-protein complex combines with it, as methemoglobin combines with H_2O_2, the compound consisting of one molecule of H_2O_2 and one molecule of peroxidase. This complex is unstable and, in the presence of any substance which can undergo oxidation, such as hydroquinone, adrenaline, or ascorbic acid, it rapidly decomposes and the original peroxidase is regenerated. It appears, therefore, that in the catalytic action of peroxidase there are two stages: *1,* the formation of an enzyme-substrate complex between ferric peroxidase and H_2O_2, and *2,* the decomposition of this compound in the presence of a suitable substrate whereby the substrate becomes oxidized by peroxide while ferric peroxidase is regenerated. H_2O_2 and phenolic substrates react very slowly, if at all, under ordinary conditions. But when H_2O_2 is combined with peroxidase it acquires great oxidizing power.

These manifold properties of iron-porphyrin compounds (transporters and stores of molecular oxygen, sluggish reversible oxidation-reduction systems, electroactive reversible oxidation-reduction systems) explain their fundamental role in cellular respiration. This fundamental role is also seen in the universality of their presence in living cells. Recognized as early as 1886 by MacMunn (38), they have been found in all aerobic bacteria (Yaoi and Tamiya, 62; Tamiya and Yamagutchi, 49; Frei, Riedmüller, and Almasy, 20); in plants (Keilin, 26; Kempner, 32); in lower animals (Roche, 43); in higher animals (Cohen and Elvehjem, 13; Huszák, 25). They have been found in organisms such as *Paramecium* (Sato and Tamiya, 45) whose respiration, according to Gerard and Hyman (21), is insensitive to HCN.

The isolation of oxidation enzymes, initiated in Warburg's laboratory, and the discovery by Warburg of the electroactive systems, the alloxazins, led some workers to attribute great significance to these systems as the last members in the series of oxidation-reduction systems found in biological

oxidations, *i.e.*, as the systems reacting directly with molecular oxygen in living cells. A warning against such rash interpretations was given by Theorell (53), who found that the rate of oxidation of hexose monophosphate in the presence of the enzyme components isolated by Warburg and his co-workers (57), namely the activating protein, triphosphopyridine nucleotide, and the yellow ferment, diminished considerably at oxygen tensions similar to those expected in multicellular organisms. On addition of cytochrome-cytochrome oxidase, the yellow ferment was reoxidized through the mediation of the cytochrome system. The same probably occurs in the oxidation of hexose monophosphate by living cells. *E. campestris,* for example, oxidizes hexose monophosphate to phosphohexonic acid as the Warburg enzyme does; the oxidation is inhibited by HCN. Furthermore, many bacteria oxidize *d*-amino acids. If alloxazin dinucleotides are part of the enzyme component in these cells, the system which transfers electrons to oxygen must be a heavy metal catalyst and not alloxazin, for the oxidation is inhibited by HCN.

A review of the considerable work done on the inhibiting effect of HCN on oxidations produced by aerobic bacteria and animal tissues would lead to the conclusion that iron-porphyrins are generally the systems concerned with the reduction of molecular oxygen. In the simplest case, as in the oxidation of lactic acid by gonococci, iron-porphyrins react directly with the oxidizable substrate when activated by the activating protein:

1) $2 \text{ lactate} + 4 \text{ Fe}^{++} \rightarrow$
$2 \text{ pyruvate} + 4 \text{ Fe}^{++} + 4 \text{ H}^+$

2) $4 \text{ Fe}^{++} + 2 \text{ O}_2 + 4 \text{ H}^+ \rightarrow$
$4 \text{ Fe}^{+++} + 2 \text{ H}_2\text{O}_2$

3) $2 \text{ H}_2\text{O}_2 + \text{catalase} \rightarrow 2 \text{ H}_2\text{O} + \text{O}_2$

where Fe^{+++} and Fe^{++} represent the series of oxidation-reductions in the cytochrome-cytochrome oxidase system. In the oxidation of hexose monophosphate, at oxygen tensions found in cells, the series of oxidation-reductions is increased by the addition of an electroactive system, alloxazin, and a sluggish system, triphosphopyridine nucleotide (T.P.N.):

1) $2 \text{ hexose monophosphate} + 2 \text{ T.P.N.} \rightarrow$
$2 \text{ phosphohexonic acid} + 2 \text{ T.P.NH}_2$

2) $2 \text{ T.P.NH}_2 + 2 \text{ alloxazin} \rightarrow$
$2 \text{ T.P.N.} + 2 \text{ reduced alloxazin}$

3) $2 \text{ reduced alloxazin} + 4 \text{ Fe}^{+++} \rightarrow$
$2 \text{ alloxazin} + 4 \text{ Fe}^{++} + 4 \text{ H}^+$

4) $4 \text{ Fe}^{++} + 4 \text{ H}^+ + 2 \text{ O}_2 \rightarrow$
$4 \text{ Fe}^{+++} + 2 \text{ H}_2\text{O}_2$

5) $2 \text{ H}_2\text{O}_2 + \text{catalase} \rightarrow 2 \text{ H}_2\text{O} + \text{O}_2$

In the oxidation of lactic acid by muscle tissues the series of oxidation-reductions is further increased by the addition of a sluggish reversible system, diphosphopyridine nucleotide (D.P.N.):

1) 2 lactate $+$ 2 D.P.N. $\rightarrow$
    2 pyruvate $+$ 2 D.P.NH$_2$

2) 2 D.P.NH$_2$ $+$ 2 alloxazin (coenzyme factor, diaphorase) $\rightarrow$ 2 D.P.N. $+$ 2 reduced alloxazin

3) 2 reduced alloxazin $+$ 4 Fe^{+++} $\rightarrow$
    2 alloxazin $+$ 4 Fe^{++} $+$ 4 H$^+$

4) 4 Fe^{++} $+$ 4 H$^+$ $+$ 2 O$_2$ $\rightarrow$
    4 Fe^{+++} $+$ 2 H$_2$O$_2$

5) 2 H$_2$O$_2$ $+$ catalase $\rightarrow$ 2 H$_2$O$_2$ $+$ O$_2$

If triose phosphate is oxidized through Szent-Györgyi's C$_4$ dicarboxylic acids, the series increases further by the addition of two enzymatic sluggish oxidation-reduction systems: oxaloacetate-malate and fumarate-succinate. Here, as in the other examples, the last member of the series, the member which reduces molecular oxygen, is the iron-porphyrin system.

As the living cell becomes more complicated, it develops a series of reversible systems of graded energies, systems which deliver smoothly the energy provided by the final reduction of oxygen. The series of oxidation-reduction systems of the cell becomes longer and longer, the iron-porphyrin compounds performing in most cases the reduction of molecular oxygen, *i.e.*, they determine the oxygen consumption, which is the fundamental characteristic of cellular respiration.

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DISCUSSION

Dr. Ball: On looking over Barron's tabulation this idea occurred to me, and I would like to ask him whether he knows of any evidence to substantiate it and its plausibility. We know that cyanide inhibits respiration, presumably by combining with a hemochromogen of a rather high potential. I don't know the potential of the cytochrome oxidase system, but presumably it lies above cytochrome-a. Now the cyanide hemochromogen systems are rather negative systems, and I am wondering whether the cyanide poisoning mechanism might be explained by cyanide combining with the cytochrome oxidase system and lowering its potential below that of cytochrome-a or cytochrome-c, which would mean that cytochromes-a and -c could not be oxidized through cytochrome oxidase and yet cytochrome-b might well remain oxidizable.

Dr. Barron: It is difficult to answer Ball's important question because we do not know the dissociation constants of the complex compound between cyanide and cytochrome. Furthermore, the amount of CN^- ions available at pH values of about 7 is very small since the pK value of HCN is 8.2. However, it is true that the affinity of CN^- for *Spirographis* hemin is greater than that for blood hemin.

Dr. Lyman: I can cite one experiment with the oxidation of fatty acids, linseed oil particularly, which may throw some light on Ball's question. We found this kind of a situation. Using hemin alone the oxidation of linseed oil was not inhibited by HCN through concentrations as high as one hundred thousandth. As we went on up to pH 8 or so, we found an inhibition. Then raising the pH still higher, up to, I believe, about 9.6, again there is no inhibition.

It seemed quite clear that HCN at pH 7 did not inhibit the action of hemin, and then at pH 9 we knew we had the formation of cyanide hemochromogen. Now the postulate was this: the

HCN inhibits not the hemin catalysis but cyanide-hemochromogen catalysis. So, to elaborate this idea we tried the effect of HCN on the inhibition at pH 7. At this pH with the concentration of HCN used, there was scarcely any CN^- left for hemochromogen formation. At pH 8 about half the cyanide is in the form of CN^-. The other half—undissociated HCN—inhibited the oxidation. So apparently it is the HCN which inhibits the reaction, and not the formation of cyanide hemochromogen.

Dr. Ball: I think you will find differences in different cases. For example, in the lactate-pyruvate system you might not find cyanide inhibition for the hemochromogen, but cyanide hemochromogen lies above the potential of the lactate-pyruvate. I think it depends on the relative position of the potential.

Dr. Barron: I think Ball's point is valid. In the first place the mechanism of the oxidation of fatty acids by hemochromogens is different from the mechanism of most biological oxidations. In the oxidation of fatty acids we have a chain reaction where the catalyst takes part only up to the moment at which a steady state is reached. In the second case we have the usual oxidation by electron transfer. The postulate that cyanide may inhibit oxidative reactions by forming hemochromogens of more negative potentials than those of the original hemochromogens cannot be dismissed without further experimentation.

Dr. Stern: Has an attempt been made to measure the potential of the *Spirographis* heminglobin complex?

Dr. Barron: Unfortunately we could not make the measurement because of the small amount of *Spirographis* hemin at our disposal. We intend to continue these measurements with pheohemin which we are preparing at our laboratory.

Dr. Stern: Is it necessary to assume on theoretical grounds that the oxidation-reduction potential of the respiratory ferment must be more positive than that of the most positive cytochrome component? Warburg a few years ago speculated on this point when he discussed the relation of the iron catalysts in *Acetobacter*. On the basis of qualitative spectroscopic observations he assigned to the iron compound No. 1 a potential considerably more negative than iron compound No. 2, ($\Delta = 180$ mv.) Such an "inverted" potential relation is, of course, only possible if the first Fe catalyst is the only autoxidisable component in the chain.

Dr. Barron: There are no theoretical grounds for assuming that the potential of cytochrome oxidase or Warburg's "*Sauerstoffübertragendes Ferment*" must be higher than that of cytochrome-a. Since in the oxidation or reduction of all these systems there is a one-electron transfer, there may be great differences in the E'_o values and yet the

system of more negative E'_o value may oxidize the system of more positive E'_o value. Such an example is found in the oxidation of lactate in muscle. The E'_o value of the reversible system lactate-pyruvate at pH 7.0 is -0.180 volt; the E'_o value of diphosphopyridine nucleotide is about -0.32 volt. The system of more negative potential (diphosphopyridine nucleotide) oxidizes the system of more positive potential (lactate).

Dr. Hellerman: I wonder if you would care to comment on the mechanism of action of catalase as depicted. It seems very remarkable in view of the fact that a variety of potent reducing agents fail to reduce catalase. When it is reduced, it seems also to be disrupted. These and other facts are a little difficult to reconcile with that mechanism.

Dr. Barron: The mechanism of catalase action given in the paper is that postulated by Keilin and Hartree. They have presented only indirect evidence in favor of their view. The fact that a variety of potent reducing agents fail to reduce catalase is no valid argument against the possibility of reducing catalase by milder reducing agents. Thus, cyanide hemochromogen is not reduced by H_2 and colloidal palladium, while it is reduced by the milder reducing system, lactate + activating protein of hydroxyoxidase.

Dr. Hellerman: Do you feel that catalase must necessarily function as a reversible oxidation-reduction system?

Dr. Barron: I think that the indirect evidence in favor of the assumption, presented by Keilin and Hartree, is worth serious consideration and fits well with the known properties of iron-porphyrin compounds.

Dr. Hellerman: You feel that is absolutely necessary?

Dr. Barron: The reason why iron-porphyrin compounds occupy such a prominent role in biological oxidations is because of their reversible oxidation-reduction. Food stuffs, in their oxidation, give H_2O_2 as one of the end products. This H_2O_2 is oxidized by an iron-porphyrin-protein complex in the same way as, let us say, succinate is oxidized by cytochrome, the reduced catalase being in its turn reoxidized by oxygen.

Dr. Hellerman: Need the catalase necessarily be reduced? May it not possibly function as catalyst in the decomposition of hydrogen peroxide without being itself reduced?

Dr. Barron: Catalase might function as catalyst for the decomposition of H_2O_2 without being reversibly reduced if H_2O_2 combines with the enzyme and this compound is disrupted with consequent decomposition of H_2O_2. Peroxidase, another iron-porphyrin-protein catalyst, acts by forming a complex component with H_2O_2. But, up to now, Keilin's arguments incline me to favor his theory for the mechanism of catalase action, name-

ly, reversible oxidation-reduction of catalase.

Dr. Stern: I agree with Hellerman that the action of catalase need not necessarily involve oxidation-reduction. The spectroscopic observations made with catalase and ethyl hydrogen peroxide may satisfactorily be explained without postulating the formation of ferro-catalase.

There is, of course, the possibility that small amounts of the reduced enzyme might exist during the stationary state of the catalase-peroxide reaction, in which case the reduced form would escape spectroscopic detection. The intermediate complex formed with the substrate appears to be a compound of the ferric enzyme and not of the ferrous enzyme.

With regard to the enzyme-H_2O_2-inhibitor complexes described by Keilin it should be noted that all the suitable inhibitors contain nitrogen (NaN_3, hydrazine, hydroxylamine). They may conceivably act not only as inhibitors but also as hemochromogen forming agents.

Dr. Melnick: It has been suggested by Barron that the oxidation of pyruvic acid proceeds through the cytochrome-cytochrome oxidase system with thiamin or thiamin pyrophosphate functioning as the hydrogen carrier. However, no definite evidence has been brought forward that thiamin or its pyrophosphate ester acts as a reversible redox system in the catalytic breakdown of pyruvic acid. Barron has found that neither reduced thiamin nor reduced thiamin pyrophosphate is biologically active, which, as he has pointed out, is to be expected in view of the reducing agent—sodium hyposulfite—which he employed. During the reduction sulfite is formed, which splits the thiamin molecule into the inactive pyrimidine and thiazol parts.

In some recent experiments carried out with Stern we have likewise investigated this problem. When hydrogen activated by platinum black is employed as the reducing agent, dihydrothiamin has no vitaminatic activity in pigeons. On the other hand dihydrothiamin pyrophosphate is just as active as the oxidized form both in polyneuritic pigeons and as cocarboxylase in the yeast test system. These findings indicate the necessity of the pyrophosphate grouping in the coenzyme; before the vitamin can undergo reversible reduction and oxidation, it must be pyrophosphorylated. This suggests to us that the pyrophosphate group acts as the connecting link between the vitamin and its protein bearer. Free thiamin is rapidly pyrophosphorylated and becomes thereby biologically active. The reduced vitamin is probably unable to undergo pyrophosphorylation, and therefore cannot join with its specific protein; it cannot then become catalytically active. But dihydrothiamin pyrophosphate already contains the chemical grouping by which it may be linked to its protein bearer and consequently is physiologically active.

Dr. Barron: Melnick's observation is indeed extremely interesting. It seems that thiamin acquires on phosphorylation the power of combining with the proteins which activate pyruvic acid, *i.e.* carboxylase and the protein of α-ketone oxidase. If we postulate that diphosphothiamin acts by combining with the protein and that this protein-diphosphothiamin complex activates pyruvic acid, there is no need for a reversible reduction of diphosphothiamin.

Dr. Barker: Is there any known chemical difference between the hemochromogen portions of myoglobin and hemoglobin that would account for the great difference in their dissociation curves?

Dr. Barron: The iron-porphyrin portions of hemoglobin and myoglobin are identical. The great difference in their affinities for molecular oxygen must be due to the nature of the protein group which unites with the iron-porphyrin.

Dr. Barker: Is there any relation between the hemochromogen or the constituent parts of the hemochromogen in the myoglobin and that in the cytochromes? Might one be derived from the other to fulfill a specialized function?

Dr. Barron: The iron-porphyrin group of hemoglobin, myoglobin, and cytochrome-c is the same (1, 3, 5, 8-tetramethyl, 2, 4-diethyl, 6, 7-dipropionic acid porphyn). One pigment may be changed into the other by combining the iron-porphyrin with the specific protein. The iron-porphyrin group of cytochromes-a, and -b, and cytochrome oxidase seems to be similar to that of *Spirographis* hemin (1, 3, 5, 8-tetramethyl, 2-formyl, 4-vinyl, 6, 7-dipropionic acid porphyn). Whether blood hemin is changed in the animal tissue into *Spirographis* hemin is not known.

Dr. Shorr: Have you any experimental evidence to localize the role of thiamin in pyruvic acid oxidation in animal tissues?

Dr. Barron: We have no definite proof of the mechanism of pyruvic acid oxidation. That pyruvic acid has to be activated by a specific protein is certain. It is also certain that diphosphothiamin is essential for both decarboxylation and oxidative decarboxylation of pyruvic acid. The mechanism of diphosphothiamin action is not known with certainty. In cells where the oxidation of pyruvic acid is insensitive to HCN (*B. Delbrückii*) it seems that alloxazin dinucleotides act as oxidation catalysts; in aerobic cells the cytochrome system is also concerned in the oxidation-reduction series.

Mr. Abrams: Do you have any experimental evidence about something that has been rather confusing to me in regard to Warburg's "*Atmungsferment*"? You have it listed in one table as active by means of electron transfer, and at the same time in the second table you have it listed with hemoglobin and myoglobin as combining with molecular O_2. You also gave a velocity constant for the latter reaction. I wonder in which class you think it belongs, and what is the experimental evidence upon which the value of the velocity constant is based.

Dr. Barron: The velocity constant is that given by Warburg. The table is that of Millikan. Since Warburg's "*Atmungsferment*" has not yet been isolated, the experimental evidence you ask for has to wait for the isolation of the enzyme. I have put Warburg's "*Atmungsferment*" provisionally among the group of iron-porphyrin compounds which act as electroactive oxidation-reduction systems, because the essential properties of the enzyme can be imitated with artificial hemochromogens.

Dr. Baumberger: I would like to ask the relative affinities of the prosthetic group for various nitrogenous molecules, and if they are such that one could account for the inhibition by cynanide on the basis Ball suggested? In the place of the hemochromogen that existed, what is the relative affinity of the nitrogenous compound for the prosthetic group? Are they known?

Dr. Ball: No.

Dr. Baumberger: Isn't it true you ordinarily have to have a relatively high concentration of a nitrogenous group to bring about a substitution?

Dr. Ball: Are you speaking about the hemochromogens made at the laboratory?

Dr. Baumberger: I am speaking of those; but I wonder if it is possible that in an organism you might have competition for the prosthetic group by cyanide at the low concentration necessary for inhibition of respiration.

Dr. Barron: If it is possible, *in vitro*, to change one hemochromogen into another by altering the concentration of the nitrogenous compounds, it is possible that such change might occur in living cells. The cyanide effect may be explained, in my opinion, by the combination of HCN with the ferri form of the iron-porphyrin, as postulated by Warburg, a combination which inhibits the electroactivity of the iron-porphyrin compound.

Dr. Hellerman: In view of Theorell's work, need we postulate that the cytochrome-c is reversibly dissociated?

Dr. Barron: The reversibility of iron-porphyrin-protein compounds exists not only among the hemochromogens made in the laboratory but also is present in hemoglobin.

Dr. Hellerman: Isn't it conceivable that in some protein-hemochromogens, for example cytochrome-c, the protein is so tightly bound that we do not have in those particular cases observable dissociation of the substituent proteins?

Dr. Barron: That is a question which cannot be answered until the other cytochromes are prepared with the same degree of purity as cytochrome-c.

REVERSIBLE PROCESSES IN THE CONTROL OF ACTIVITY OF CERTAIN ENZYMES

WITH A PRELIMINARY NOTE ON THE OXIDATION OF UREASE BY PORPHYRINDINE

Leslie Hellerman

Investigators in the field of enzymatic catalysis thoroughly agree that reliable results are predicated upon the rigorous control of experimental conditions. Although proper temperature control, for example, is in any branch of catalysis a first consideration, the choice of working temperatures in enzymatic catalysis must take special account of the lability of the catalysts. Again, there was little systematic progress until the ampholytic nature of most enzymes was recognized. Then adequate methods were devised for the control of hydrogen ion activity. The growing recognition of the protein character of many enzymes, culminating in the isolation of several crystallized protein-enzymes, resolved certain difficulties and afforded a satisfying theoretical basis for certain procedures. However, our limited knowledge of the structures of proteins remains a vexing handicap—and a challenge.

None of the peculiarities of enzymatic behavior has presented in recent years a more troublesome problem than the management of specific effects often introduced by the presence of heavy metal ions, of foreign proteins, and of such extractives as glutathione and other accompanying materials. In certain instances, indeed, attention has been directed to interactions with the oxygen of the air. Inductive studies of these specific effects taken with systematic observations of the effects of various reagents purposely introduced into reaction mixtures, have led to useful generalizations regarding reversible chemical processes of importance in the control of activity of certain biological catalysts. Such catalysts include representatives of the hydrolytic enzymes, of reductases, and even of hemolysins and lysozymes. Examples of the reversible processes are the salt-forming (*e.g.*, mercaptide-formation), the metallo-coördinative, and the oxidative-reductive. The latter category is of chief concern to this paper. Several reviews with extensive bibliographies are available (1, 2). Therefore no attempt will be made here to approach completeness in any aspect of the discussion.

For quantitative biology the subject holds a three-fold significance. It implies, in the first place, a better appreciation of the factors concerned in the experimental treatment of biological catalysis. It offers, in the second instance, suggestions concerning the control of catalysis *in vivo*. It presents, finally, valuable hints for the investigation of the nature and the structure of the enzymes themselves.

That certain oxidizing agents suppress and certain reducing agents enhance the activity of urease or papain is now an old story. The rather capricious nature of the effects of these reagents (upon the enzymes concerned) is characteristic. Omnipresent oxygen may for a considerable period leave unscathed the urease molecules in a solution of the recrystallized protein-enzyme or in a crude preparation containing protecting contaminants derived from the jack bean. Nevertheless, the presence of a little iron salt, or better a minute amount of cupric-ion, in the solution of crystalline urease, permits the oxygen more rapidly to wipe out the catalytic activity. The inhibition may be reversed quantitatively by the addition of hydrogen sulfide, cysteine or reduced glutathione. Iodine-iodide mixtures may be added to urease or to papain under such conditions as to cause ready abolishment of the enzymatic activity, again restorable, however, in appreciable measure by reducing agents such as thiol compounds, hydrogen sulfide, hydrogen cyanide, or (in the case of papain) even titanous chloride. However, ascorbic acid anion and numerous other reductants may fail to restore or enhance the activity of partially inactive or reversibly inactivated preparations. The system $Fe(CN)_6^=:Fe(CN)_6^≡$ even in high concentration fails to bring about the appreciable inactivation of purified urease in solution during a period of hours at room temperature. But ferricyanide does inactivate papain (or certain hemolysins (*loc. cit.*)) at 37° C., its effect being reversed by suitable reducing reagents. The oxidants of such rapidly reversible oxidation-reduction dye systems as methylene blue or indophenols are often found to have little observable effect. However, the activity of pneumococcal hemolysin is depressed more or less rapidly by such oxidants. One aspect of the peculiarities of these effects is revealed in a consideration of the relative free energies of oxidation-reduction of some of the reagent systems used in these studies; the potentials of the half-reduced systems at about pH 7 are listed in Table I.

From the clear-cut evidence of reversible inactivation by processes of oxidation-reduction as well as from certain apparent inconsistencies revealed in systematic tests, and from other evidence, there appeared cogent support for a practical working hypothesis. Certain aspects of the behavior of the pertinent bio-catalysts were assumed to be explicable in terms of reversible chemical reactions with substituent sulfhydryl groups of

TABLE I

The potentials of certain oxidation-reduction
systems

System	E'₀ at pH 7
	volt
1 1/2 I₂ : I⁻	ca. *0.7
2 Fe(CN)₆⁼ : Fe(CN)₆≡	0.44
3 Phenolindophenol	0.228
4 2, 6-dichlorophenolindophenol	0.217
5 Ascorbic acid	0.060
6 Methylene blue	0.011
7 The simpler sulfhydryl compounds** (e.g., cystine : cysteine)	—0.3
8 Ti⁺⁺⁺⁺ : Ti⁺⁺⁺	<—0.3 (?)

* In dilute solution, roughly as used in these studies.

** A rough approximation; the results of different workers vary owing to experimental difficulties and uncertainties.

the protein-enzyme molecules. The argument has been detailed elsewhere (1) and need not be developed here. Chemical evidence, admittedly of an indirect sort, provided the basis. For a schematic representation, applicable to the behavior of appropriate enzymes, we may consider the equation:

$$2 \text{ En-S-H} \rightleftharpoons \text{En-S-S-En} + 2 \text{ H}^+ + 2 \epsilon$$
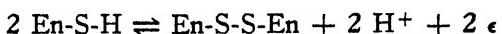
(It is conceivable that the change might take place intramolecularly, for example, if two -SH groups of a single protein molecule were involved.) The form, En-S-H, denotes the catalytically active "sulfhydryl-enzyme" and En-S-S-En, the inactive (reversibly) oxidized enzyme molecule. However, the equation is not to be taken as representing a rapidly reversible system, the characteristics of which, as a thermodynamically reversible oxidation-reduction process, are readily determined by equilibrium methods. It is well recognized that mercaptan-dithio "systems", although reversible in a general chemical sense, are more or less sluggish in their behavior. They are notoriously sensitive to the action of certain catalysts. Both reductant and oxidant components possess a versatile reactivity; for example, the oxidants (R-S-S-R) under well-defined conditions are subject to "hydrolytic" disproportionation changes (e.g., to yield mercaptan and sulfonic acid). It is an interesting fact that the thiols, which from all available evidence are reductants of relatively "negative" systems, are most conveniently estimated with the use of oxidizing agents of the

potency of ferricyanide, iodine, or porphyridine, with which they react rapidly.

With regard to the validity of the sulfhydryl idea, the existence of tissue-proteins containing detectable sulfhydryl groups (3) apparently supplied direct support of a general character. Especially was this true of the demonstration (4) that urease is associated with sulfhydryl groupings that respond to a nitroprusside test. Later I shall attempt a preliminary evaluation of this kind of evidence.

An interesting consequence of the sulfhydryl idea is associated with the action of "mercaptide-forming" reagents such as various heavy metal salts, cuprous oxide, and organic mercury compounds of the type R-Hg-X (where X is -OH or -Cl, as illustrated in p-chloromercuribenzoate ion, ClHg-C₆H₄-COO⁻). These have been found readily and characteristically to cause inactivation of urease, papain, certain hemolysins, etc., the effect being reversed by the use of reagents favoring the splitting of mercaptides. While it has not been proved definitely that protein mercaptides are concerned, and, in certain instances there has not been ruled out the possibility of a "surface effect," the weight of evidence appears to support the thiol-mercaptide picture. In those cases where the active protein molecules appear to contain nitroprusside-detectable thiol groups, the test is abolished by the addition of the heavy metal compounds. Moreover, metallic reagents which cannot form mercaptides, such as dibenzyl mercury or even bis-iodomethyl mercury, have little observable effect. Studies with certain suitable arsenic compounds (2) have yielded results consistent with the foregoing.

An obvious corollary of these interpretations concerns the protective action or actual enhancement of catalytic activity frequently observed in the presence of various reagents. Examples are hydrogen sulfide, hydrogen cyanide, and pyrophosphates. The effects obviously are not always to be associated with reductive activation; there may also be concerned the removal of specific metallic catalysts of oxidation (of the enzymes) as well as of the salt-forming heavy cations. No reference is made here to the more general salt effects. It must be emphasized also that the reagents regarded here as activators may in other well defined fields of biological catalysis assume totally different properties. Thus, Kubowitz's polyphenol oxidase is a copper-protein; arginase, unusual among the better known hydrolytic enzymes, possesses apparently a metallic constituent; certain phosphorylations are promoted by magnesium and manganous ions; and the prothrombin-thrombin conversion is aided by calcium-ion. There are other interesting cases. Manifestly,

those reagents which decompose or dissociate metallo-enzymes, or which remove specific metallic coenzymes, are depressant in their action. Moreover, bio-catalysts of the metalloporphyrin-protein type may be inhibited through coordinative association (5, 6) with donor molecules such as carbon monoxide, hydrogen cyanide, and numerous nitrogenous bases. The association constants vary over a wide range. The role of the equilibria in the control of catalysis of biological oxidations is another and fascinating story.

If time permitted, it would be of unusual interest to recount the order of events which have advanced the field of the biologically interesting thiol compounds. We should then be struck with the abundance of observations as well as with the versatility of biological function assigned to such substances. Immediate emphasis, perhaps, would be given glutathione, cysteine, ergothioneine, and the sulfhydryl proteins and protein-catalysts. No less compelling is the interest in "related" dithio substances, including keratins and globular proteins with constituent -S-S- linkages, among which insulin (7, 8) has received intensive study.

Glutathione is of wide-spread occurrence in cells; it can be contained abundantly in plant and animal tissues in which are found also activatable bio-catalysts. Most workers now agree that the dithio forms are very readily reducible by substances of mercaptan character including reduced glutathione; and, indeed, are subject to rapid and extensive activation by the tripeptide. It is difficult, however, to assign specific functions to the substance. An exception appears in its role as "coenzyme" for glyoxalase, in which its combination with the substrate appears to be an essential step. Concerning a specific function for ergothioneine, even less is known. It has been suggested to the writer that this thiolglyoxaline may, in the red cells for example, serve a protective function through antioxidant properties.

It is of interest that the properties of sulfhydryl-containing protein molecules also are assuming marked prominence in current investigations. This may be attributed more particularly to two developments. The one centers in the recent intensive interest in the nature and structure of globular proteins, together with the development of more powerful methods of investigating protein dissociation and "denaturation". The other stems from the correlations of reversible inactivation of a number of bio-catalysts with reversible processes affecting supposedly integral sulfhydryl and disulfide groupings. From a structural viewpoint the two fields are related. The latter field, however, has assumed a rather surprisingly broad biochemical importance. In its sphere have been gathered, in addition to urease, papain and certain hydrolases accompanying cathepsins, the following: crystallized ficin, certain lipid-splitting enzymes, several pneumococcal and streptococcal hemolysins, and lysozyme of egg-white.

More recently Ecker and his colleagues (9) have indicated that complement of the immunologists is to be added to those principles which apparently fit the thiol-dithio hypothesis. Controlled oxidation depresses activity; reduction by cysteine, dithionite, or hydrogen sulfide restores activity. A correlation has been shown also between the concentration of blood ascorbic acid in the guinea pig and the activity of the complement. The role of sulfhydryl groups in appropriate antigenic enzymes is receiving scrutiny. Under investigation, also, are the thiol substituents of crystalline virus proteins (10).

Elsewhere (1) we have emphasized that the available evidence concerning the role of substituent -SH groups has dealt almost exclusively with a *regulatory* function—a mode of control of activity and an extension of factors requiring consideration in experimental work. This is no less applicable to the observations in several different laboratories which concern the oxidative-reductive regulation of certain principles having, themselves, a function in biological oxidation-reductions. Such principles are those associated with the succinate-fumarate systems, and with the process of glycolysis in appropriate tissue preparations. Hopkins and Morgan (11) demonstrate that protein in preparations of succinic dehydrogenase carries nitroprusside-detectable thiol groups which are readily oxidizable. They show, further, that the activity of the reductase is suppressed by the use of sufficient oxidized glutathione, and that the inactive, washed enzyme is rendered active again by treatment with reduced glutathione. This recalls the experiences of several workers who find that mixtures of sluggish thiol-dithio systems interact and may appear to approach an equilibrium state (12, 13). Hopkins and Morgan show, moreover, that the phenomenon, as observed, does not involve a glutathione-specific process. Suitable oxidants are depressors of activity; appropriate reductants, activators (*cf.* 14). Presumably a sulfhydryl-disulfide equilibrium, as such, does not concern directly the mechanism of action of succino-dehydrogenase, for the disulfide form is inactive and is not reduced by succinate.

A study of reversible inhibition by oxidation and reduction in systems of importance to glycolysis was made by Lipmann (15). The subject has received scrutiny by several workers (16). The more recent work of Rapkine (17) has been characterized by the examination of a protein-enzyme which functions in connection with an oxidative-reductive equilibrium of central import-

ance in anaerobic processes. Omitting for present purposes consideration of any accompanying phosphorylation process, the potential interaction between the ions concerned may be stated thus:

$$\text{triosephosphate} + \text{pyruvate} \rightleftharpoons \text{phosphoglycerate} + \text{lactate}.$$
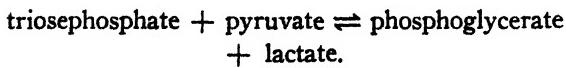
Granting the validity of current conceptions of the glycolytic process, it appears that an important element in the displacement of the equilibrium to the right (when oxygen is absent or the catalysts which permit its efficient functioning are dormant) is the irreversibility of the final step in a series of seemingly intramolecular transformations which carry phosphoglycerate to non-phosphorylated pyruvate. The equilibrium may be regarded as involving two ordinarily unreactive oxidation-reduction systems, the triosephosphate-phosphoglycerate and the pyruvate-lactate, respectively; each requires its own specific protein-enzyme virtually to elicit a latent and potential reversibility. Mediating between the two systems, permitting rapid interaction, is a third oxidation-reduction system, a catalyst system, which *under the conditions* is rapidly reversible, namely pseudospecific cozymase. For the protein-enzyme which functions with the first-named substrate pair, designated in one system of nomenclature as triosephosphate dehydrogenase (of muscle), there is now at hand presumptive evidence which supports the applicability of the thiol-disulfide hypothesis. This evidence appears to assign to the thiol-disulfide equilibrium no role other than a regulatory one. It will be recalled that Gemmill and Hellerman (16) presented a clear-cut picture of reversible inhibition which they tentatively interpreted in terms of an oxidative-reductive control of catalysis of the glycolytic mechanism in frog muscle. Leövey (18), in our laboratory, found that the process governing conversion of pyruvate to lactate in rabbit muscle pulp is suppressed by certain oxidants and restored by hydrogen sulfide. Rapkine now takes a critical step in an attempt to localize the site of control.

With regard to newer developments concerning the methods available in dealing with protein thiol groups, only two technical matters need be considered here. The first concerns the recent work upon addition compounds of maleic acid with mercaptans (19). Succino-dehydrase has been shown to be inhibited irreversibly by the ions of maleic acid. There is thus available another welcome diagnostic tool which may have value so far especially as the more reactive protein thiol groups are concerned. Contrarily, iodoacetate is being more and more distrusted as such a diagnostic reagent. There is, of course, no question that iodoacetic acid and related reagents can under

proper conditions act as alkylating agents toward thiols. There is, however, serious question about their specificity in connection with the thiol groupings of native proteins, and particularly of protein-enzymes subject to the thiol-disulfide activation picture. For example, iodoacetate appears to inactivate papain and perhaps other bio-catalysts reversibly—a result hardly to have been anticipated on an assumption of thiol alkylation. It is true that reagents of this class have been found occasionally to bring about oxidations (20). However, iodoacetate inhibits succinic dehydrogenase but slowly and, conversely, suppresses drastically some of the reductases to which the thiol relation has been found inapplicable (*e.g.,* aldehyde reductase). Actually, we know altogether too little about the specificity of behavior of various reactive substances with protein molecules. For this reason, the interpretation of observations in this field had best retain the element of caution.

We have discussed again interesting evidence disclosing reversibility in the inhibitory actions upon certain enzymes of heavy metal salts and of oxidizing agents. On the basis of substantially indirect evidence, we have entertained the suggestion that there may be involved, in a limited number of cases, reversible chemical actions upon substituent sulfhydryl groups of the active proteins. It is appropriate now to consider, in relation to this problem, the very limited information permitting an appraisal of the relative reactivity of protein thiol groupings.

In several instances, some of the substituent thiol groups of native proteins have appeared to be readily detectable. However, substituent groupings are not always immediately detectable in presumptive tests. Obviously, the qualitative protein tests often involve drastic manipulations which injure or decompose the proteins. With regard to the possibility of differences in the reactivity of individual groupings, the sulfhydryl residues have attracted unusual attention recently, doubtless because of their great inherent reactivity and the seeming specificity of their action with oxidizing agents, derivatives of iodoacetic acid, and other reagents. Groupings occurring in protein "side-chains" often appear to be easily detectable. Examples are the lysine ε-amino groups and, in insulin, the amino groups of phenylalanine residues, detected by Jensen with the aid of phenyl*iso*cyanate.

It is common experience that the denaturation of protein molecules, whether by heat, agitation in solution (surface effect), the action of certain chemical reagents or otherwise, is usually accompanied by effective accentuation of the reactivity of various characteristic groupings. These may

include a portion of such disulfide and thiol groups as were substituents in the native protein (21).

For the estimation of protein thiol groups, a number of methods have been proposed. A convenient reagent is the deeply colored, reactive oxidant, porphyrindine (22, 23, 24, 25) which appears in fact to have some applicability for proximate estimation.
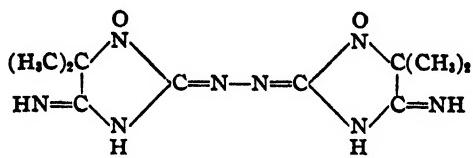
Porphyrindine

This, and the related substance, porphyrexide, constitute oxidants of rapidly reversible oxidation-reduction systems which, thermodynamically, stand among the most powerfully oxidizing of the organic systems. With porphyrindine, V. E. Deitz and I have atttempted to study further the characteristics of urease. Our findings, although reasonably clear-cut, must be considered as strictly preliminary, because we were hampered during our investigation with difficulties in regard to the availability of jack bean flour of the quality necessary for the production of crystalline urease. This section of the paper is essentially a progress report. I have selected for discussion a few of our more suggestive results.

A solution of once recrystallized urease was titrated with thousandth molar aqueous porphyrindine. The more readily detectable (4) sulfhydryl groups were oxidized rapidly. There was obtained a "semi-permanent" end-point; the nitroprusside test, initially strong, was now found to

be negative. Since the concentration of active protein enzyme, as revealed by activity tests in comparison with total nitrogen content, approached but did not reach that of the most active preparations (27), we cannot be certain that the thiol groups titrated were exclusively substituent labile groups of active molecules. For example, denatured urease (27) may have contributed. If for present purposes, however, we assume that 2 -SH groups are oxidized by a molecule of porphyrindine, we calculate that the apparent equivalent weight of urease per sulfhydryl group residue weight titrated is roughly 30,000. Be that as it may, we were greatly surprised to find, upon test, that the partially oxidized urease retained unimpaired its original catalytic activity. This active, titrated urease was readily inactivated by p-chloromercuribenzoate, from which activity was restorable by the action of sulfhydryl glutathione. The results are illustrated in Table II.

Table II shows also that enzymatic activity was retained after the addition to untitrated enzyme of a rather appreciable quantity of a mercury compound capable of forming mercaptides (experiment 3). With this, the results of experiments 4 and 5 provide an interesting contrast. The subject will be placed upon a more quantitative basis when suitable working material shall have become more available. Any calculations are for illustrative purposes only. We desire to avoid reading into our data a possibly fictitious quantitative significance.

To portions of urease (in phosphate, pH 7) was added porphyrindine *seriatim* in increasing increments, but always in excess of an amount required to remove the initially more reactive -SH groups. The enzyme was readily and progressively inactivated. In contrast to the effect of

TABLE II

Behavior of urease with porphyrindine and other reagents.

Expt.		Solution	Activity (units (26) per ml. urease)
1	A	Urease in phosphate buffer, pH 7 (initial)	2900
2	B	A* plus 0.4 ml. 0.001 M Po. per ml.	3050
3	C	A " 1.0 ml. 0.001 M Hg.	3000
4	D	B " 1.0 ml. 0.001 M Hg.	190
5	E	D " 0.5 ml. 0.01 M Gl. (pH 7)	2900
6	F	B " 1.0 g. guanidine hydrochloride* per ml.	70

* Solution A, after treatment with 1.0 g. guanidine hydrochloride per ml. required 1.7 ml. 0.001 M porphyrindine.

Po. denotes porphyrindine; Hg. potassium p-chloromercuribenzoate; Gl., reduced glutathione.

iodine or oxygen (plus catalysts), this inactivation, under the conditions, appeared to be irreversible to a very appreciable extent (oxidation beyond the -S-S- stage?). It may be noted that porphyrindine possesses a somewhat varied reactivity. In the hands of Kuhn and Desnuelle, Greenstein, and others it appears to have served advantageously in approximate oxidimetric titrations. For example, sulfhydryl groups or "potential" sulfhydryl groups of egg albumin, initially unreactive with porphyrindine, may in part be titrated after the protein has been denatured with guanidine hydrochloride, etc. (24). However, the reagent has not yet been shown to convert protein thiol sulfur exclusively to the dithio configuration; indeed, a critical study of its action upon simple sulfur compounds seems to be lacking. In some of our titrations, there were formed colored products which possibly may have contained addition compounds.

Of interest, further, are the observations illustrated in Table III. From these it appears that

TABLE III

Covering effect of p-chloromercuribenzoate.

Exp.		Material tested	Relative activity
1	A	urease (initial)	870
2	B	A + Hg.	32
3	C	B + Gl.	900
4	D	A + Po.	455
5	E	D + Gl.	405
6	F	D + Hg.	11
7	G	F + Gl.	385
8	H	B + Po.	11
9	I	H + Gl.	845

Po.; Hg.; Gl.: See Table II.

a partial irreversible inactivation through the agency of porphyrindine does not hinder a superimposed reversible inactivation by p-chloromercuribenzoate. Contrarily, the urease, "covered" (reversibly) with the mercurial, is protected effectively against the action of the indicator.

An additional observation was the following. The protein denaturant, guanidine hydrochloride, was observed to destroy the activity of urease, the extent of activity loss depending upon the concentration of the salt. In the presence of 1 gram per ml. (Table II, exp. 6) the enzyme is substantially inactive. The addition of the guanidine salt brings on display reactive sulfhydryl groups greatly in excess of those initially titratable. Of these, one would be the group directly titratable

with porphyrindine (and apparently not concerned with activity); a second, that attacked in a subsequent reversible inactivation by mercury compound after the initial titration (Table II). We have not yet tested with dialysis procedures or otherwise the reversibility of the guanidine effect, which strongly suggests a denaturation. It will be recalled that the protein constituent of Warburg's "first" yellow enzyme may be separated so that the protein is rendered denatured (with the appearance of -SH groups?); further, that the effect in this instance can be reversed (Theorell; cf. 23).

These preliminary observations may offer suggestions aiding the elucidation of certain puzzling aspects of our problem. Assume that the available evidence may be taken to indicate that immediately reactive, possibly peripheral, thiol groupings of the urease molecule are concerned neither in the catalytic mechanism, nor in the oxidative inactivation of the enzyme. The basis for the sulfhydryl theory still remains; it may be asked whether, if thiol groupings are indeed concerned, these are not in fact derived from a separate category of less reactive, "masked," thiol groups (21), characteristic of the native protein molecules. Such thiol groupings, whether or not loosely combined within the protein molecule, seem to share with other characteristic substituents (-S-S-, HO-C_6H_4-, etc.) the property of being rather unreactive. Their relative unavailability to reagents in certain instances is related very probably to the nature of their orientation within the molecule. On this basis it is not surprising that so drastic a change as "denaturation" may alter their reactivity. In the case of urease, the pertinent groupings concerned would be included among such as are found to be readily oxidizable after, but not antecedent to, denaturation. In native protein molecules, a diminished reactivity of thiol groups, with respect to certain reagents thermodynamically qualified to react, for example ferricyanide and dye oxidants such as the indophenols, might be attributable simply to their relative physical inaccessibility to the action of these reagents. This becomes especially significant when it is recalled that many of the reagents thus devoid of immediate potency, are found often to belong to the classification of those materials considered especially prone to form loose chemical combinations with proteins.

These interpretations, based upon protein structure, are not to be taken as in disparagement of other factors which may condition the rate of interaction of mercaptans with oxidizing and other reagents. In such processes, the role of catalysis and anticatalysis is too well appreciated to require discussion. It is well recognized, too, that the

mercaptans display among themselves great variations in their apparent reactivity with various reagents. Group effect has its part. However, these factors, alone, do not suffice for the present problem, which appears to involve additional, and new, dimensions.

Such considerations may bear, for example, upon apparent anomalies presented by papain. Unusually beautiful consistency with the -SH: -S-S- activation picture is exhibited by papain; nevertheless it is extremely difficult to obtain even with crude, potent preparations a direct demonstration of sulfhydryl substituents. Papain has been found to be a relatively stable enzyme (1), not readily susceptible to some of the denaturation procedures. Is it not conceivable that in native papain we are dealing with the structurally less accessible -SH groups, the protein being actually destitute of the immediately reactive, "surface" variety?

The case of hemolysins or lysozymes may present a different situation. Certain hemolysins seem to be unusually reactive with respect to their sulfhydryl substituents. The activity of pneumococcal hemolysin is rather readily suppressed by oxidant components of a large series of dye systems (28). This is in marked contrast to the behavior of papain or urease. Is it not altogether probable that the activation picture applied to certain lytic substances of this character concerns, not inaccessibly bound groupings, but rather the immediately accessible variety? Evidence that such sulfhydryl groups enter *directly* into the mechanism of lytic action is not at hand. It seems entirely possible that the oxidation of the thiol groupings in these cases may interfere in a secondary way, preventing characteristic orientations with cell surfaces. The assumption of any specific role for a reactive, catalytically unimportant thiol fraction of urease would be gratuitous at present.

In this sketch our principal concern has centered on the examination of a type of reversible process which may enter, through an oxidation-reduction mechanism, into the regulation of the pace of certain biological processes. It may be emphasized that there exists no compelling evidence assigning to sulfhydryl groups, which have entered so prominently into the discussion, any specific function in the catalytic mechanisms, themselves. It is readily conceivable, however, that they do so participate—possibly in collaboration with other protein groupings.

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DISCUSSION

Dr. Anson: Hellerman found some years ago that urease is not inactivated by ferricyanide. Smythe found later that urease is inactivated by iodoacetic acidamide. Native albumin does not react with ferricyanide, but, as I have observed recently, some of the —SH groups of native egg albumin react with iodoacetic acidamide. This result fits in with the urease result.

As Hellerman mentioned, crude extracts of animal tissues digest gelatin. The reagents which inhibit papain in neutral solution inhibit the digestion of gelatin. The reagents which activate papain in neutral solution promote the digestion of gelatin. But the inhibition and activation of the gelatin-digesting enzymes take place at pH 3.5. I should like to ask Hellerman to discuss how these reactions in acid solutions can be explained on an —SH basis.

In the study of papain, when the enzyme is activated enzymatically and not by reagents such as cyanide, the practical problem arises of how to estimate the papain without inactivating it. For instance, if a 30 per cent suspension of commercial papain is allowed to stand for several days at 37° C. in the presence of toluol there is a considerable activation, but if one attempts to estimate the activated papain by the digestion of hemoglobin, then there is inactivation again by traces of heavy metals in the dilution water and in the hemoglobin solution. This inactivation can be prevented by dilute neutral cyanide, which by itself cannot activate the original papain. In general, when impure papain and impure substrate are used, the interpretation of activation and inactivation experiments is extremely uncertain. Thus when papain is inactivated reversibly by iodacetic acid, the iodoacetic acid may not have reacted with the papain protein at all. Only in experiments like Hellerman's with pure urea and pure urease is it possible to study both the enzyme activity and the enzyme protein and thus to obtain results of definite significance.

Finally, I should like to ask Hellerman what happens to cysteine and SH proteins when an excess of porphyrindine is added.

Dr. Hellerman: My reply to the last question must be that I do not know exactly. I suppose porphyrindine could do a number of things. Under certain conditions, it might oxidize sulfhydryl groups beyond the dithio stage, possibly even to —SO$_3$H. Until we know more about the chemistry of the action of porphyrindine on various groupings I would rather defer any extended discussion.

Dr. du Vigneaud: Do you get a good endpoint when you titrate beyond this?

Dr. Hellerman: If one adds a denaturant first and then buffers, one gets an end point that might not satisfy the requirements of exact analysis, but which seems fairly indicative for some purposes. I am not particularly happy about the use of porphyrindine as an analytical reagent, but I think it may serve some very useful purposes. I have used it chiefly to eliminate the "reactive" —SH groups. Anson's discussion illustrates some of the difficulties of experimentation and also of interpretation; I did not go into practical points of that sort in this paper because I thought I had treated adequately some of these points in a review paper in 1937. I quite agree regarding the pertinence of many of these questions. The use of a reagent such as urease simplifies the problem considerably. I am looking forward to seeing what Lineweaver and Balls will have found in connection with the oxidation and reduction of their crystalline papain.

In this connection I want to mention the very significant studies of du Vigneaud, of Jensen, and of Stern and White on the activation of insulin, which has not been proved to be an enzyme but is certainly a protein hormone. In many respects the inactivation chemistry is quite different from that of urease; nevertheless there are certain relations which might have been discussed in a more extensive paper. One of the most significant relations is concerned with the observation that only one or a very few of the -S-S- linkages of the insulin molecule need to be reduced in order to wipe out activity. What the ultimate significance of facts of that sort may be I do not know, but they are highly interesting facts.

Dr. du Vigneaud: In your experience with urease have you found any difference between the impure and pure material in their bahavior? Was all of your material crystalline?

Dr. Hellerman: All the work I have published on the reversible inactivation of urease has been with purified (crystallized) urease. In the case of papain it was non-crystalline material, of course.

Dr. du Vigneaud: Have you done any work with the non-crystallized urease?

Dr. Hellerman: Very little. Of course the literature records a number of investigations with such material.

Dr. du Vigneaud: Have you found any differences?

Dr. Hellerman: There are significant differences, and so great that I cannot always interpret them. As a matter of fact, it was because of the difficulty of interpretation of the older results with impure urease and impure papain, etc., that I had the temerity to tackle the problem. With the purified material the picture has been nicely consistent; the interpretations may be somewhat shaky, but the experimental results have been consistent.

Dr. Anson: When you inactivate an enzyme such as urease by a heavy metal such as silver or mercury and then make the solution acid, does the heavy metal stay attached to the protein?

Dr. Hellerman: We have not done work of that sort with urease or papain as thoroughly as we should have. In certain cases involving enzymes it is fairly easy to dialyze away some of the heavy metals.

Dr. Stern: Hellerman mentioned the work of Theorell, who observed that upon reversible dissociation of the yellow enzyme by weak HCl the protein moiety gave a positive nitroprusside test and was incapable of reuniting with the prosthetic group. Only when the "metaprotein" was "renatured" by dialysis against distilled water, could the coupling with the flavin-phosphate be accomplished.

I think one might mention at this point that Kuhn and Desnuelle repeated the experiment of Theorell and were unable to confirm his observa-

tions. It is in this paper that the porphyrindine method was first put to a test.

Mr. Furchgott: Have any determinations been made upon the molecular weight of an enzyme such as urease in the inactivated oxidized form? Such determinations might show whether the oxidation involved an inter-molecular or intra-molecular reaction of sulfhydryl groups.

Dr. Hellerman: I do not think so.

Dr. Anson: It is not known in the case of any protein whether in the oxidation of two -SH groups to an S-S group, the two -SH groups are provided by a single protein molecule or by two different protein molecules.

Dr. Stern: But it has been done with insulin. White and I used insulin treated with thioglycollic acid and sent it partially reduced to Svedberg, and he found that the molecular size had not been affected by the partial reduction.

Dr. Hellerman: I pointed out a relation between insulin and urease, but in so far as the point under discussion is concerned, the case of insulin has not much to do with urease. It is a very interesting point chemically, but is it significant biologically?

Dr. du Vigneaud: I think that Anson has reference to the fact that the molecular weight of the oxidized form has not been determined. The analogy to the insulin work would not be the molecular weight of the reduced insulin, but the molecular weight of the reduced and reoxidized insulin. The question is whether upon reoxidation the sulfur from various molecules were linked together.

Dr. Anson: In fact, that might be the explanation of why the inactivation is not reversible.

Dr. du Vigneaud: In the first paper on the reduction of insulin we pointed out that the failure to get reactivation on reoxidation might have been due to the fact that one could not expect to get an appreciable amount of the original molecule back.

Dr. Anson: Can you titrate these groups so as to separate the heavy metal compounds?

Dr. Hellerman: I should be much surprised if one could, because I should consider such reactions to be reversible. The answer would depend upon the magnitude of the equilibrium constants, and I do not know what they are.

Dr. Anson: When you inactivate urease by a heavy metal, is the amount of heavy metal you add equivalent to the number of -SH groups you believe to be abolished?

Dr. Hellerman: I find that for action apparently upon a particular, unique group in the urease molecule, one mole of RHgX reagent would be required to inactivate very roughly 30,000 grams of the enzyme.

Dr. Anson: Is that true of the other heavy metals?

Dr. Hellerman: It would depend upon the metallic reagent used. That is a rather important point, because compounds of the type of RHgX are a little more specific with protein sulfhydryl groups at pH 7 than, for example, silver ion.

THE EFFECTS OF VARIOUS METALS AND METAL COMPLEXES ON THE OXIDATION OF SULFHYDRYL GROUPS

Frederick Bernheim and Mary L. C. Bernheim

This discussion will be confined to the effect of certain metals and metal complexes on the oxidation of the sulfhydryl group. In the first part, the oxidation of simple compounds such as cysteine, glutathione and thioglycollic acid will be considered; in the second part, the oxidation of the sulfhydryl groups of proteins; and in the third part, the enzymic oxidation of cysteine and thioglycollic acid. The emphasis will be laid on the differences in the reactions brought about by different agents on the various sulfhydryl compounds.

1.

A. Literature

Cysteine. Since the discovery of the effect of iron on the oxidation of cysteine by Mathews and Walker (1) and Warburg and Sakuma (1a), the catalytic effect of many other metals has been tried. Bauer and Preis (2) showed that copper, manganese and mercury were also effective as catalysts, but that cyanide, iodide, cobalt, lead, cadmium, zinc, nickel, uranium, vanadium, platinum and gold inhibited the autoxidation. Michaelis and Barron (3) made nickel, cobalt and iron complexes of cysteine and studied their oxidation potentiometrically. Krebs (4) showed that hemin could act as a catalyst for the oxidation of cysteine and that the oxidation was very sensitive to cyanide. Johnson and Voegtlin (5) made stable arsenic derivatives of cysteine. Schubert (6, 7) isolated iron and cobalt complexes of cysteine and their carbonyl derivatives. According to Rosenthal and Voegtlin (8) copper oxidizes cysteine causing decarboxylation and deamination, whereas iron and manganese oxidize it to cystine. Elvehjem (9) found copper sixteen times as active as iron for the oxidation of cysteine and explained Gerwe's (10) results on traces of copper present in his preparations. According to Schöberl (11) the iron catalysis is more inhibited by acid than the copper catalysis.

Glutathione. For glutathione, Voegtlin, Johnson and Rosenthal (12) showed that copper and hemin were active catalysts but inorganic iron was inactive. They extended these results in a subsequent paper (13) and added palladium, cobalt, selenium, tellurium and gold to the active group, whereas manganese, nickel, tin, lead, cerium, mercury, chromium, osmium and arsenic were inactive. Lyman and Barron (14) showed that the effect of copper increased with increasing pH and had no optimum, whereas hemin had an optimum at pH 8.0. They found the hemin catalysis relatively insensitive to HCN compared to the hemin catalyzed oxidation of cysteine.

Thioglycollic acid. The oxidation of thioglycollic acid by metals was studied by Thunberg (15). He found manganese a better catalyst than iron or cobalt. Kharasch et al. (16) showed that the copper-catalyzed oxidation had an optimum at pH 6.0 and that combinations of manganese and copper were more active than either alone. Phosphates inhibited the manganese and iron catalysis but accelerated the copper. Bersin (17) found that arsenic, copper, gold, antimony, tin and iron in this order were active in oxidizing various aniline derivatives of thioglycollic acid.

Miscellaneous oxidizing agents. According to Holtz and Triem (18), Schales (19), and Schöberl (11) hydrogen peroxide is produced when sulfhydryl compounds are oxidized. The effect of copper and iron on the oxidation of cysteine and glutathione by H_2O_2 was studied by Pirie (20) who found the iron catalysis partially inhibited by phosphate and completely inhibited by pyrophosphate. He also showed (21) that thiocarbamide and dithioformamidine oxidize cysteine in the presence of H_2O_2. Ghosh and Kar (22, 23) used vanadic, tungstic and molybdic acid sols with H_2O_2 to oxidize cysteine and thioglycollic acid.

Preisler and Preisler (24) oxidized cystine to cysteic acid with thallic sulfate and found that small amounts of iodide accelerated the oxidation but chlorides and bromides inhibited it. Toennies and Lavine (25) oxidized cystine to cysteic acid by perchloric acid in non-aqueous media. Labes and Freisburger (26) showed that alloxan accelerated the oxidation of thioglycollic acid anilide and (27) that alloxan combined with sulfhydryl groups. This was shown also by Lieben and Edel (28). The effect of the presence of disulfide at the beginning of the reaction in accelerating the rate of oxidation was shown by Dixon and Tunnicliffe (29) for cysteine and Kharasch et al. for thioglycollic acid (16). Harrison (30) showed that dithioglycollic acid accelerated the oxidation of sulfhydryl groups both aerobically and anaerobically. Andrews (31) found that cystine was oxidized to cysteic acid after prolonged standing in air. Sulfate inhibited the oxidation. The literature on the action of some of the more common metals as inorganic salts on the oxidation by oxygen of cysteine, glutathione and thioglycollic acid is summarized in Table I.

B. Experimental

Because we have been studying the biological action of vanadium it was of interest to compare the effects of this metal with those of iron and copper on the oxidation of sulfhydryl compounds.

TABLE I

Metal	Cysteine	Glutathione	Thioglycollic Acid
Copper	+++(2) (8)	+++(12) (14)	+++(16)
Iron	++(1)	−(12)	++(15)
Manganese	+(2)	−(13)	++(15)
Mercury	+(2)	−(13)	−(17)
Arsenic	−(5)	−(13)	+++(17)
Cobalt	−(3)	+(13)	+(15)

These metals make complexes with 8-hydroxyquinoline and the effects of the complexes were compared with those of the inorganic salts. 8-Hydroxyquinoline, according to Kolthoff (32), has an ionization constant of $10^{-4.7}$ and an isoelectric point at pH 7.2. The metals, according to Berg (33), replace the hydrogen on the hydroxyl group and may have some secondary valence linkage with the nitrogen. Montequi and Gallego (34) isolated various vanadium complexes. The other isomers of hydroxyquinoline do not make this type of complex with metals and the effects described below are only obtained with 8-hydroxyquinoline. 2-Hydroxyquinoline and quinoline gave negative results.

The final concentration of the metals was about 4×10^{-3} and of 8-hydroxyquinoline was 7×10^{-4}. This was found to be in the middle of the optimum concentration range for the activity of the complex. These complexes are formed immediately on mixing the metals with hydroxyquinoline. The vanadium complex is deep yellow and the iron complex is dark purple. The vanadium was used as sodium metavanadate, although vanadium acetate was equally effective. The copper was used as copper sulfate, the iron as ferrous sulfate and manganese as manganese chloride. It made no difference in what order the metal or hydroxyquinoline was added to the sulfhydryl compounds or whether the complex was made before the addition. All the experiments were carried out in M/20 phosphate buffer. Controls showed that the effect of the metals or their complexes was not altered by this concentration of phosphate with the exception of manganese which was slightly inhibited.

The cysteine hydrochloride, thioglycollic acid and reduced glutathione were obtained from Hoffmann-LaRoche. The oxidation of these sulfhydryl compounds without the addition of a metal was negligible below pH 7.0 under the conditions of the experiments. Mixtures of vanadium with copper or iron showed that the vanadium effect was not mediated by traces of these metals which

may have been contaminating the sulfhydryl compound and conversely that copper and iron were not acting with vanadium. The specificity of the metal effects also excludes the possibility of an interaction between two metals being responsible for the effects observed on the addition of one.

The effect of pH on the oxidation of the sulfhydryl compounds by the metals and their hydroxyquinoline complexes was measured. As is well known, the effect of copper and iron increases with increasing pH although the extent of this increase varies with the sulfhydryl compound. Thus the difference in the rate of oxidation of thioglycollic acid at pH 6.0 and 7.5 by iron is small compared with the difference in the oxidation rate of cysteine at these two hydrogen ion concentrations. In the case of thioglycollic acid where vanadium is an active catalyst, the rate of oxidation decreases with increasing pH, but manganese on the other hand is only active above pH 7.0. The hydroxyquinoline, where it has a definite effect on the metal oxidation, shows a maximum between pH 6.0 and 7.0. Below pH 6.0 all the effects decrease.

The results which are shown in Fig. 1 a and 1 b may be summarized as follows (because the glu-
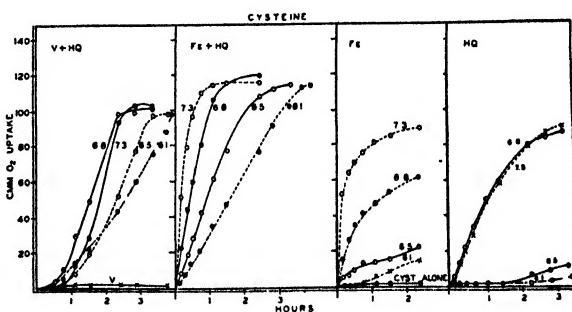
Fig. 1 a. The oxidation at various hydrogen concentrations of 4.0 mg. cysteine hydrochloride by various metals with and without 7.0×10^{-4} M 8-hydroxyquinoline and by 8-hydroxyquinoline alone. Sodium metavanadate was present in a concentration of 4.1×10^{-3} M and ferrous sulfate 3.6×10^{-3} M. 37° C.
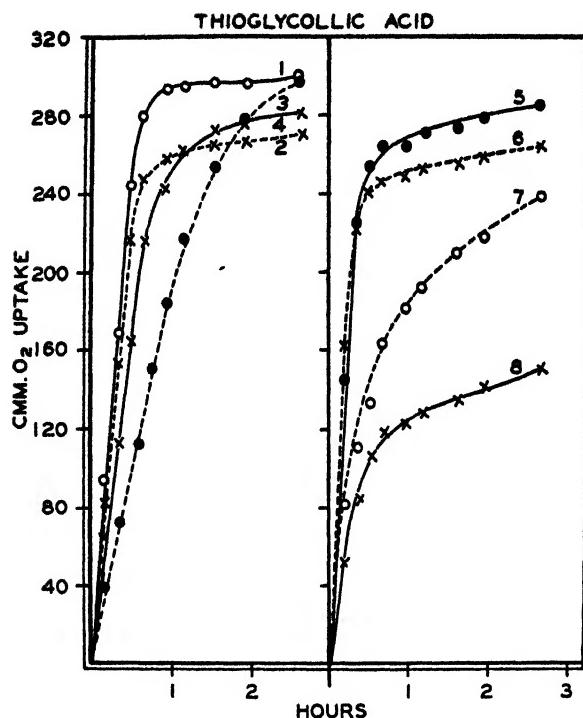
Fig. 1 b. The oxidation of 4.0 mg. thioglycollic acid by various metals with and without 7.0×10^{-4} M 8-hydroxyquinoline. The metals were used in the same concentration as in Fig. 1a. The pH was 6.1 except for manganese which was done at pH 7.5. The manganese sulfate was used in a concentration of 4.2×10^{-3} M. The numbers on the curves represent the following: 1: Cu, 2: Cu + HQ, 3: V + HQ, 4: V, 5: Fe + HQ, 6: Fe, 7: Mn + HQ, 8: Mn.

tathione results, with the exception of copper, are all negative the curves are not given). Thioglycollic acid is oxidized by ferrous sulfate, copper sulfate, sodium metavanadate and manganese chloride. 8-Hydroxyquinoline alone has no effect on the oxidation and in the presence of the metals tends to inhibit the rate of oxidation by vanadate and accelerates the oxidation by manganese. It has no effect on the copper and iron catalysis. Cysteine is oxidized by iron and copper and slowly by manganese, but vanadium is without effect. 8-Hydroxyquinoline alone has a definite effect on the oxidation between pH 6.8 and 7.5. Above and below this range its effect is negligible. It is therefore difficult to conclude anything about the effect of the metal hydroxyquinoline complexes in this pH range. But at pH 6.1 and 6.4 the effects of the vanadium and iron hydroxyquinoline complexes are much greater than the effects of the constituents alone. The latent period in the vanadium complex effect is characteristic and occurs also in the case of the protein described below.

The oxygen uptakes of these compounds show that they are oxidized to the disulfide form with the exception of cysteine catalyzed by copper, which is oxidized further.

II.

A. Literature

Some native proteins contain sulfhydryl groups. These include myosin (35, 36), hemoglobin and the protein of the crystalline lens (37) and the proteins of jack bean urease (38). Many other proteins show sulfhydryl groups on denaturation. This was first shown by Hopkins (39) and later studied by Mirsky and Anson (37). Recently Greenstein (40), using the porphyrindin dye introduced by Kuhn and Desnuelle (41), has estimated the number of sulfyhydryl groups liberated after various denaturing agents. After urea has acted on egg albumin, sulfhydryl groups equivalent to 1 p.c. cysteine are liberated, and treatment with guanidine liberates the equivalent of 1.24 p.c. Heat denaturation liberates about half this number. These results indicate that a protein reacts specifically to different agents. Burk (42) postulates cyclic and straight chain sulfur linkages to account for different degrees of stability of amandin and excelsin in urea solutions.

B. Experimental

It was of interest to see whether the sulfhydryl groups of proteins could be oxidized by metals and hydroxyquinoline. Tissue proteins were examined first. Depending on the tissue and animal, the addition of 8-hydroxyquinoline and a metal to a tissue suspension may cause a large increase in oxygen uptake. If such a suspension, made by grinding the tissue with sand and squeezing through muslin, is brought to pH 6.7 by the addition of M/20 phosphate buffer and centrifuged, an insoluble protein fraction comes down. This can be suspended in water with some phosphate buffer (pH 6.7) and centrifuged again. After the third centrifuging the protein is practically free of hemoglobin, is light yellow in color and by itself takes up no oxygen. If vanadium and hydroxyquinoline are added to this protein suspension a large oxygen uptake occurs. Vanadium or hydroxyquinoline alone is without effect.

Investigation of this phenomenon showed that liver, kidney, brain, muscle, pancreas and tumor tissue contained the protein which could be washed by centrifuging several times and still remained its activity. For most of the following experiments the protein from rat liver was used. In order to determine what constituent of the mixture was being oxidized concentration curves were made. These are shown in Fig. 2. Within limits, the amount of oxygen taken up is independent of the amount of vanadium or hydroxyquinoline present, but is dependent on the amount of protein. This indicates that the protein is being oxidized and
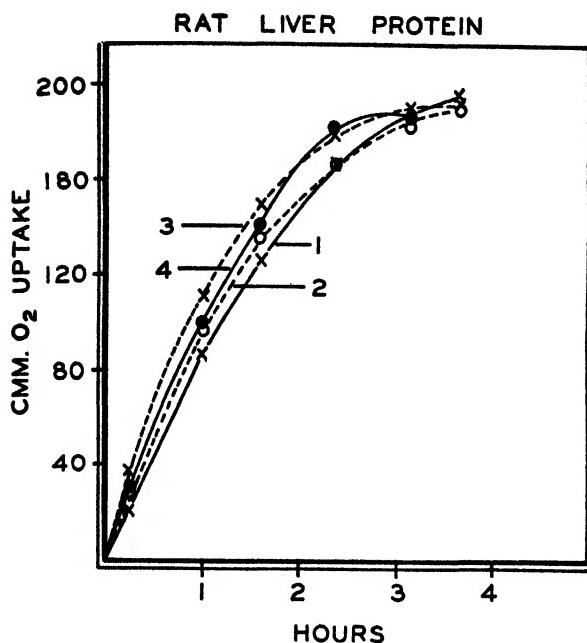
RAT LIVER PROTEIN

Fig. 2. The effect of various concentrations of sodium metavanadate and 8-hydroxyquinoline on the oxidation of rat liver protein. pH 6.5. 37° C. The numbers on the curves represent the following: 1: 0.1 cc. V + 0.1 cc. HQ, 2: 0.05 cc. V + 0.3 cc. HQ, 3: 0.05 cc. V + 0.2 cc. HQ, 4: 0.1 cc. V + 0.2 cc. HQ.

that the vanadium-hydroxyquinoline complex is the catalyst. Iron is also effective in bringing about this oxidation. In this case the iron alone has a small effect which is greatly increased by the hydroxyquinoline but the final uptake of oxygen is only about half of that which takes place in the presence of the vanadium complex. Copper and manganese alone or with hydroxyquinoline are without effect.

Properties of the protein. During the oxidation no ammonia or carbon dioxide is liberated. This suggested either that the hydroxyl groups of tyrosine or serine or that sulfhydryl groups were being oxidized. Experiments with tyrosine and serine were negative and the positive results obtained with cysteine suggested that the sulfhydryl groups

were attacked. The nitroprusside test was used to demonstrate the presence of sulfhydryl groups. On the washed protein from liver this test was found to be strongly positive and disappeared after oxidation with the vanadium or iron hydroxyquinoline complexes. Other proteins were then tried. Serum proteins which have no free sulfhydryl groups before or after boiling were not oxidized in the presence of a metal complex, nor was insulin or casein. Crude and crystalline egg albumin gave negative nitroprusside tests and even though sulfhydryl groups appeared after boiling or treatment with urea no oxidation occurred. Jack bean urease, which has a large number of free sulfhydryl groups, was not oxidized. This showed that free sulfhydryl groups were a necessary but not a sufficient condition for the oxidation. The protein extracted from the eggs of the speckled trout (*Cynoscion nebulosus*) was highly active. It was prepared by grinding the eggs with sand and dissolving them in dilute sodium hydroxide at pH 8.3, centrifuging off the insoluble material, and precipitating the protein from solution with acetic acid at pH 5.5. This process could be repeated many times with no loss of activity. The product was pure white, and gave a marked nitroprusside test which became negative after oxidation.

The conclusion from these experiments was that only protein derived from within the animal cell and containing free sulfhydryl groups was susceptible to this oxidation, and suggested that the protein was a nucleoprotein. Accordingly phosphorus determinations were done on the protein of the liver and fish eggs in various stages of purification. The liver protein, after three washings by centrifuging, was dialyzed overnight to get rid of free phosphate. It was then dissolved and precipitated by the process described above for the fish eggs. This was repeated twice. The results are shown in Table II. As the phosphorus-nitrogen ratio increases the amount of oxygen taken up per milligram of protein increases, showing that in both cases it is a protein with a high phosphorus content which is the specific substrate for the oxidation.

The question arises whether the protein thus obtained from liver and fish eggs is native or de-

TABLE II

Liver Protein	P/N	O_2 uptake per mg. protein mm.^{3}	Fish egg protein	P/N	O_2 uptake per mg. protein mm.^{3}
Washed 3 times	0.070	10	1 ppte	0.143	15
+ 1 ppte	0.088	22	2 ppte	0.166	20
+ 2 ppte	0.106	24	3 ppte	0.196	21

natured. There is no doubt that after successive dissolving and precipitation, denaturation takes place as the protein becomes progressively less soluble. In the case of the liver the question is hard to answer because the mere breaking up of the cells may be sufficient to bring about denaturation. For the fish eggs it is easy to show that the original untreated eggs have a marked nitroprusside reaction and after dissolving the protein in alkali and precipitating with acid only a small part of the material giving the test remains in solution. This indicates that the positive nitroprusside test on the untreated eggs was not due to cysteine, glutathione or similar small molecules. There is therefore a strong presumption that the native phosphoprotein of the fish eggs contains free sulfhydryl groups and this may also be true of the liver protein.

If these phosphoproteins are treated with alcohol, acetone or dioxane, they are no longer oxidized by the metal complexes although the sulfhydryl groups are still present. Boiling for ten minutes partially inactivates the liver protein, but has no effect on the fish egg protein. Treatment with urea and subsequent removal of the urea leaves the protein in an oxidizable state although the rate of oxidation is somewhat slower. Precipitation of the liver protein from the tissue suspension with dilute hydrochloric acid rather than phosphate buffer gives an inactive product. Too long exposure to acid or alkali also inactivates it. The necessary and sufficient conditions before a protein is susceptible to oxidation by these metal complexes are as follows: it must have free sulfhydryl groups, it must have a high phosphorus content, and it must not be subjected to treatment with organic solvents, or remain too long in contact with strong acid or alkali.

The properties of the oxidation. Fig. 3 shows

Fig. 4 a. The effect of 4.1×10^{-3} M sodium metavanadate, 3.6×10^{-3} M ferrous sulfate and 4.0×10^{-3} M copper sulfate with and without 7.0×10^{-4} M 8-hydroxyquinoline on the oxidation of rat liver protein at various hydrogen ion concentrations and 37° C.

that the oxygen uptake in the presence of vanadium and hydroxyquinoline is a function of the protein concentration. In neither case does vanadium alone have any effect. Fig. 4 a and b compares the effect of the vanadium and iron com-

Fig. 3. The effect of 4.1×10^{-3} M sodium metavanadate and 7.0×10^{-4} M 8-hydroxyquinoline on the oxidation of different concentrations of rat and fish egg protein pH 6.1. 37° C.
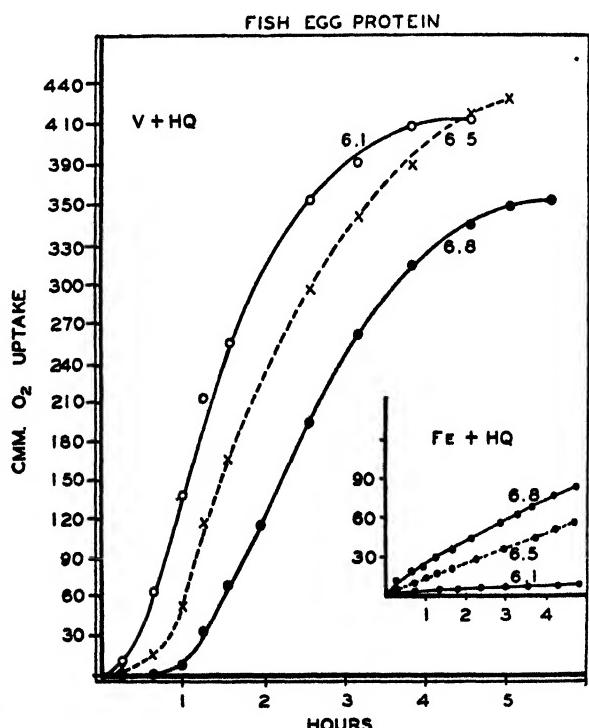
Fig. 4 b. The effect of 4.1×10^{-3} M sodium metavanadate and 3.6×10^{-3} M ferrous sulfate with 7.0×10^{-4} M 8-hydroxyquinoline on the oxidation of fish egg protein at various hydrogen ion concentrations and 37° C.

plexes at various pH's on the liver and fish protein. The iron complex is more effective on the liver than on the fish protein. For both proteins the vanadium-catalyzed oxidation shows a definite latent period which occurred also with cysteine. This latent period becomes shorter as the pH decreases from 6.8 to 6.1. The amount of oxygen taken up is somewhat less at the higher pH. Iron alone causes a very small uptake in both proteins (shown for the liver protein in Fig. 4a). Copper alone or with hydroxyquinoline has no effect on the liver protein, but copper alone rapidly oxidizes the fish protein and hydroxyquinoline inhibits this oxidation to some extent. A comparison of the rates of oxidation of the various metals and complexes on the fish protein is shown in Fig. 5. This copper effect resembles

that described by Rosenthal and Voegtlin (43) on boiled egg albumin. Manganese with or without hydroxyquinoline has no effect on either protein in acid or alkaline solutions.

In all cases where oxidation occurs the nitroprusside test becomes negative showing that the sulfhydryl groups are being oxidized. The proteins contain 1 - 2 p.c. sulfur and the oxygen uptake per milligram of fish egg or rat liver protein in the purest preparations with vanadium and hydroxyquinoline is around 20 mm³. This uptake is too large even if all the sulfur is oxidized to sulfonic acid. It is therefore necessary to conclude that some other group in the protein is also attacked, but it is still unknown what this group is.

III

With the exception of the action of copper on cysteine, the metals and their complexes catalyze the oxidation of simple sulfhydryl compounds by oxygen to the disulfide form. It has been assumed for a long time that cysteic acid is a precursor of taurine, and the body can produce it by the oxidation of the sulfhydryl group of cysteine to sulfonic acid. Various oxidizing agents such as thallic acid (24) can oxidize both cysteine and cystine to cysteic acid but the literature contains no reference to a tissue catalyst that can bring about this oxidation.

If cysteine is added to the rat liver suspension at pH 6.7, a rapid oxygen uptake occurs. The amount taken up varies but is always greater than can be accounted for by the oxidation of cysteine to cystine. In order to investigate this phenomenon, the liver suspension was washed by centrifuging at pH 6.7 with the addition of a small amount of M/20 phosphate buffer. After two such washings the insoluble protein which had centrifuged down each time was suspended in phosphate buffer, pH 6.7. Seven grams of rat liver made a final suspension of 10 to 12 cc. This protein suspension is practically free of hemoglobin, is yellowish in color, and has no appreciable oxygen uptake. If cysteine is added to this suspension an oxygen uptake occurs which is again greater than the theoretical uptake for the production of cystine. As cystine itself was not oxidized by the preparation at pH 6.7, the results suggested that in the presence of the protein, part of the cysteine was oxidized to cystine and part was oxidized further by a different mechanism. If cysteine is added to liver protein which has been boiled for five minutes the oxygen uptake is equivalent to the oxidation to cystine. In other words, the extra oxygen uptake can be eliminated by boiling. This result indicates that cysteine added to liver protein undergoes two types of oxidation; an oxidation to cystine by a thermostable catalyst,
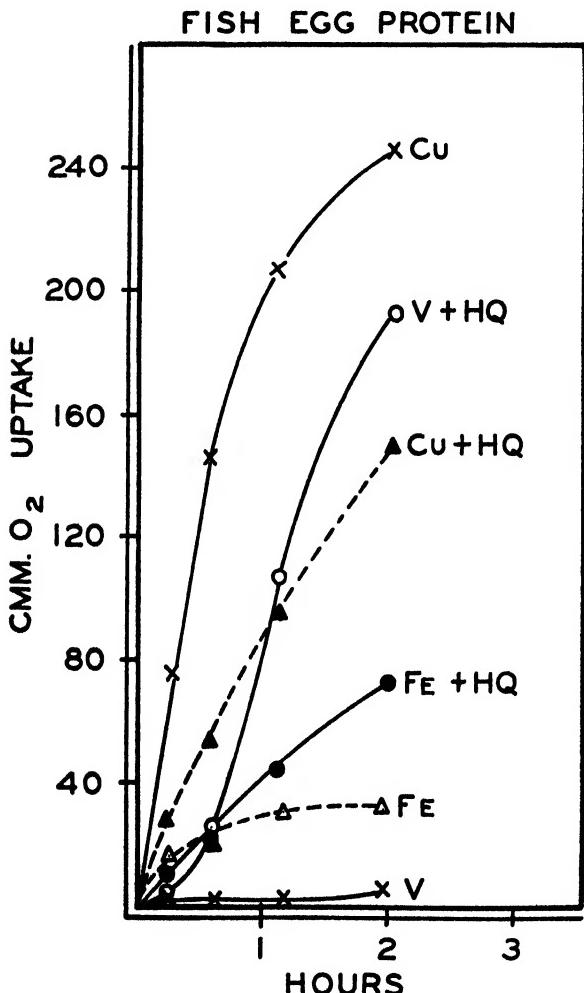
Fig. 5. The effect of 4.0 × 10⁻³ M copper sulfate, 4.1 × 10⁻³ M sodium metavanadate and 3.6 × 10⁻³ M ferrous sulfate with and without 7.0 × 10⁻⁴ M 8-hydroxyquinoline on the rate of oxidation of fish egg protein at pH 6.1 and 37° C.

probably iron or copper, and an oxidation involving more oxygen by a thermolabile catalyst.

As the oxidation of cysteine to cystine by traces of metals proceeds slowly in acid solutions it seemed possible to adjust the protein and cysteine concentrations so that the more rapid oxidation by the thermolabile catalyst would occur to the practical exclusion of the slow metal catalysis. This condition was attained by using 0.4 to 0.6 cc. of the liver protein suspension and 0.25 to 0.5 mg. of cysteine hydrochloride in each Warburg vessel at pH 6.7. This volume was made up to 2 cc. with M/20 phosphate buffer. Under these conditions a rapid oxygen uptake occurs which stops sharply when exactly three atoms of oxygen are taken up per molecule of cysteine. As no deamination or decarboxylation accompanies this reaction the oxygen uptake corresponds to the quantitative production of cysteic acid. This oxidation is shown in Fig. 6 (Curve 1).

Fig. 7 (Curves 4, 5 and 6) shows that a similar reaction takes place with thioglycollic acid. In both cases the formation of a strong acid can be shown by carrying out the oxidation in the absence of buffer. Under these conditions there is a definite shift in pH of 0.2 - 0.3 of a unit to the acid side. The enzyme, however, does not oxidize all sulfhydryl groups. Glutathione is not oxidized in this way, and in the presence of the enzyme it goes slowly to the disulfide form. Ethyl

Fig. 7. The oxidation of different amounts of thioglycollic acid (neutralized) with 0.4 cc. of washed liver protein at pH 6.7 and 37°. The curves represent the oxygen uptake of the liver and the thioglycollic acid, and the liver, thioglycollic acid, and 6 micrograms of titanium, as sodium pertitanate, from which the oxygen uptake of the respective controls has been subtracted. Curve 1, liver, 0.5 mg. of thioglycollic acid, and titanium; Curve 2, liver, 0.25 mg. of thioglycollic acid, and titanium; Curve 3, liver, 0.125 mg. of thioglycollic acid, and titanium; Curve 4, liver and 0.5 mg. of thioglycollic acid; Curve 5, liver and 0.25 mg. of thioglycollic acid; Curve 6, liver and 0.125 mg. of thioglycollic acid. The horizontal lines represent the theoretical values for the uptake of 3 atoms of oxygen per molecule of thioglycollic acid.

Fig. 6. The oxidation of 0.25 mg. of cysteine hydrochloride (neutralized) with 0.4 cc. of washed liver protein at pH 6.7 and 37°. Curve 1, the oxygen uptake of liver and cysteine hydrochloride from which is subtracted the uptake of the liver alone; Curve 2, the oxygen uptake of liver, cysteine hydrochloride, and 6 micrograms of titanium as sodium pertitanate from which is subtracted the uptake of the liver and titanium alone. Horizontal line A, theoretical uptake for 3 atoms of oxygen per molecule of cysteine hydrochloride; horizontal line B, theoretical uptake for the oxidation of cysteine hydrochloride to cystine.

mercaptan is only very slowly attacked by the enzyme and no definite end points could be obtained.

Recently Medes (44) has shown that liver and kidney slices can oxidize cysteine with the production of sulfate. This represents the further oxidation of cysteic acid and the ability to carry out this step is lost when the tissue is broken up and the enzyme purified by washing. In a private communication Medes states that she finds that cystine is oxidized by the enzyme to cysteic acid more rapidly than cysteine and therefore postulates cystine as an intermediate in this oxidation. She worked at pH 7.6. As stated above, cystine is not oxidized at pH 6.7, so it is possible that at the higher pH the mechanism of the oxidation is somewhat different.

The effect of metals. The effect of sodium pertitanate prepared as already described (45) on the enzymic oxidation of cysteine and thioglycollic acid is shown in Fig. 6 (Curve 2) and 7 (Curves

1, 2 and 3). This salt of titanium has a small accelerating action on the oxidation of cysteine alone but inhibits the enzymic oxidation completely. In the case of thioglycollic acid the inhibition tends to wear off after a length of time which is dependent on the original concentration of thioglycollic acid. This indicates that the inhibition is reversible. With cysteine the inhibition varies with the concentration of enzyme present as shown in Table III. The effect of other metals on this enzymic oxidation is seen in Table IV. Manganese as manganese chloride or potassium permanganate, and cobalt as cobalt sulfate inhibit completely at very low concentrations. Chromium salts cause a slight inhibition and vanadium and nickel are without effect. This inhibition by manganese and cobalt cannot be due to complex formation with the cysteine for the inhibition is the same whether 250 or 500 gamma of cysteine are oxidized in the presence of 2 - 3 gamma of these metals. Small amounts of KCN completely inhibit the enzymic oxidation of cysteine and thioglycollic acid.

TABLE III

Effect of 6 micrograms of titanium as sodium pertitanate on oxygen uptake of 0.25 mg. of cysteine hydrochloride with varying quantities of washed rat liver protein at pH 6.7 and 37° C.

Time	0.2 cc. liver + cysteine hydrochloride		0.4 cc. liver + cysteine hydrochloride		0.8 cc. liver + cysteine hydrochloride	
	Alone	Titanium	Alone	Titanium	Alone	Titanium
min.	*mm³. O₂*	*mm³. O₂*	*mm³. O₂*	*mm³. O₂*	*mm³. O₂*	*mm³ O₂*
10	1	2	8	0	33	9
20	3	5	20	3	54	14
30	12	9	27	4	60	21
40	19	15	33	8	59	25
50	23	16	37	7	61	24
60	25	18	40	10	62	23
70	28	23	46	14	61	24
80	32	26	50	13	63	24
90	33	29	52	14	60	24

The theoretical value for the uptake of 3 atoms of oxygen per molecule of cysteine hydrochloride is 54 mm³.

TABLE IV

Effect of manganese chloride, cobalt sulfate, nickel chloride, potassium dichromate, chromium potassium sulfate, and sodium metavanadate on oxygen uptake of 0.25 mg. of cysteine hydrochloride (neutralized) in presence of 0.5 cc. of washed liver protein at pH 6.7 and 37° C.

Time	Alone	2.5 micrograms Mn	2.2 micrograms Co	2.5 micrograms Ni	3.5 micrograms Cr, $(K_2Cr_2O_7)$	3.0 micrograms Cr, $(Cr_2K_2(SO_4)_4)$	20.0 micrograms V
min.	*mm³. O₂*	*mm³. O₂*	*mm³ O₂*	*mm³. O₂*	*mm³ O₂*	*mm³. O₂*	*mm³ O₂*
15	25	2	2	27	5	15	26
30	41	6	5	41	14	20	44
45	51	8	7	45	25	25	53
60	54	8	8	51	29	28	55
90	56	6	7	56	37	29	57
135	55	4	6	58	39	30	53
155	53	4	4	59	42	32	54

The figures given in micrograms are the amounts of metal present in the salts added. The total volume of fluid in each vessel was 2.0 cc.

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DISCUSSION

Dr. Fleischmann: Is anything known of the importance of vanadium in the tissue respiration of the tunicates?

Dr. Bernheim: Henze was unable to show that it had a function in these animals. Regarding the function of vanadium in the mammal there has been no work except from the pharmacological standpoint. There are indications in the literature that small amounts of vanadium added to the diet definitely increase the health and growth of the animal, and where vanadium has been used therapeutically for various infections, the results are interesting but not clear-cut.

Dr. Barker: Is there vanadium in the animal body?

Dr. Bernheim: Yes. It is possible to estimate it chemically. When vanadium is fed, there is an increased concentration in the liver, and small increases in other parts of the body.

Dr. Stern: Concerning the occurrence of vanadium in tunicates it might be interesting to mention that T. F. Fulton, who worked on the colored vanadium-containing particles in the blood cells of these animals, found that vanadium is apparently present in purely inorganic form, as the oxide. Henze has speculated on the role of vanadium; he believes that vanadium has something to do with the synthesis of cellulose, which occurs uniquely in these organisms.

Concerning Bernheim's very interesting experiments with vanadium and 8-hydroxyquinoline, I presume that he expected to find a strong inhibition of the vanadium effect. I think his experiments demonstrate clearly that by a tight complex-formation the catalytic property of a heavy metal need not necessarily be inhibited.

Dr. Bernheim: There is the question of how these complexes catalyze this oxidation. They might possibly act as a dye such as porphyrindin. We tried the effect of oxidizing and reducing agents on these complexes. They are highly colored, and if they are oxidized or reduced you might expect a change in color. You get it in the case of the iron complex in particular. Both oxidation and reduction bleach the color, but so far we have been unable to restore the original. Therefore, although these complexes may be oxidized or reduced, the process in not readily reversible. The stability of the complex is quite great. However, on heating, internal rearrangements probably occur because the complex precipitates and the color changes.

Dr. Velick: In your oxidations of cysteine, did you follow the rate of disappearance of the sulfhydryl groups?

Dr. Bernheim: Yes; it disappears. In the presence of titanium it does not disappear.

Dr. Velick: The experiments indicating cys-

teic acid formation are extremely interesting. There is some evidence that suggests that in the biological synthesis of taurine the cysteine first combines with cholic acid and the coupled product is then oxidized and decarboxylated to taurocholic acid.

Dr. Bernheim: Yes; that has been the hypothesis, but these tissues are able to oxidize free cysteine readily. The oxidase is cyanide-sensitive and I don't know yet whether it goes through the indophenol oxidase-cytochrome system or not.

Dr. Velick: There has been no indication of free cysteic acid in normal tissue.

Dr. Bernheim: No; I don't know whether it has been looked for. After feeding cysteic acid, nobody has ever reported a high percentage of it in urine.

Dr. Salomon: It has been shown by Schueler in Warburg's laboratory that potassium-ferricyanide not only oxidizes the ferrous iron in the prosthetic group of hemoglobin to the ferric form but oxidizes also the SH groups of the globin part of the molecule. The degree of oxidation taking place depends on the time during which the globin and the oxidizing agent (ferricyanide) are in contact with each other. It is probable that different oxidizing agents oxidize the hemoglobin molecule to a different degree and that therefore different ferrihemoglobins can be produced. Have you tried to oxidize hemoglobin with the oxidizing agents discussed in your paper, and do these agents produce catalytically active methemoglobin, when added to intact non-nucleated red blood corpuscles?

Dr. Bernheim: No.

Dr. Burk: In regard to vanadium, it plays a very decided and definite role in *Azotobacter* metabolism. In fact, *Azotobacter* cannot be grown without vanadium or molybdenum. The latter is indeed better than vanadium, but vanadium will in general act seven-tenths as well. The function of these elements is concerned in some way or other with the utilization of different kinds of nitrogen and in particular nitrogen compounds which must be reduced, such as free nitrogen or nitrate. So far we have not been able to detect any effect on the utilization of ammonium salts and we have obtained a smaller effect on the utilization of nitrate as compared with N_2, but this might even be a matter of the concentrations needed, which in any case are exceedingly small. Thus the maximum effect can be obtained at something like 10-100 parts per trillion. Quite possibly molybdenum or vanadium may be playing a role in the nitrogen metabolism or organ-

isms generally, but of course is not detected as yet, because of the very small concentrations involved.

Dr. Bernheim: At your suggestion I tried molybdenum compounds to see whether they had action similar to vanadium, and could find nothing in this particular case. One of the actions of vanadium has to do with the oxidation of the unsaturated bonds of phospholipids, which is quite different from nitrogen fixation. Molybdenum has very little effect on the oxygen uptake of the mammalian tissue.

Dr. Burk: I forgot to say that this effect of molybdenum and vanadium has been observed in other organisms besides *Azotobacter* but somewhat related to it, like the legume bacteria.

Dr. Stern: Isn't it true that vanadium and molybdenum are very effective catalysts in the inorganic form in the Haber-Bosch ammonia process?

Dr. Burk: Yes, but so are about fifteen elements. So, from our point of view, the roles in the bacterial and Haber processes are probably rather incidental.

Dr. Barker: Is there any possibility that the metals have something to do with the sulfur metabolisms of these organisms?

Dr. Burk: It is entirely conceivable.

Dr. Hellerman: It seems to me that Bernheim's work again illustrates the importance of protein structure in connection with the oxidation of the protein molecule. I refer particularly to the question of orientation of groupings (*e.g.*, —SH) within the protein molecule. I hope Bernheim will continue his interesting studies in relation to the properties of proteins and in experiments in which the egg albumin and similar molecules are denatured by chemical reagents.

It is of interest that some of the metals Bernheim has been talking about are very potent activators of arginase.

Dr. Bernheim: Does arginase require the SH group?

Dr. Hellerman: In my opinion there is no evidence that it does.

Another interesting point is this. I believe you said that manganous-ion did not function very well in the acid range, but only in the more alkaline range. This seems to be general support for the idea that you are dealing with metallic complexes. For example, the manganous-amino acid complexes are relatively more highly dissociated than the corresponding cobaltous complexes. Manganous salts activate arginase very strongly under slightly alkaline conditions, but not in the more acid range.

CHEMICAL REACTION OF THIAMIN AND COCARBOXYLASE *IN VIVO*

M. A. Lipton and C. A. Elvehjem

The wide distribution of thiamin in all types of cells, and its apparent role in the physiology of the cell has naturally stimulated considerable research regarding its specific role *in vivo*. Various approaches to the problem have been made, and each study has yielded definite information.

It was known quite early that some relationship exists between thiamin and carbohydrate metabolism, for Funk (1) reported more than twenty years ago that the thiamin requirement of pigeons decreased as the carbohydrate content of the ration was decreased. These studies have been repeated more recently with improved rations and have yielded very clear-cut results. Thus, Stirn, Arnold, and Elvehjem (2) have shown that a rat brought to polyneuritis upon a high carbohydrate ration deficient in thiamin, may be cured and caused to grow when the carbohydrate is isocalorically substituted in this ration by fat. Arnold and Elvehjem (3) have likewise shown that the nutritional requirement for thiamin in the case of rats, chicks, and dogs may be accurately expressed in terms of the carbohydrate content of the ration. The low thiamin requirement of these varied species in the presence of a ration high in fat leads to the conclusion that the vitamin is not required for the catabolism of fat. On the other hand, the correlation between the thiamin requirement and carbohydrate intake suggests that the vitamin is essential for some phase of carbohydrate metabolism.

Historically, the foundation for the further knowledge of the mechanism of thiamin action lay in these early nutritional studies upon the relation of thiamin to the carbohydrate content of the ration. But more specific information regarding the action of this vitamin has been achieved with the aid of the manometric technique, either in the study of surviving tissues *in vitro,* or of isolated enzyme systems.

In 1931, Gavrilescu and Peters (4) determined the oxygen uptake of brain from normal and avitaminous pigeons, *in vitro,* and concluded that the respiration in the presence of glucose was significantly lowered in thiamin deficiency. Later it was observed that the addition of a few micrograms of thiamin concentrate to the Barcroft flask produced a definite increase in the oxygen uptake of polyneuritic pigeon brain in the presence of lactic acid. Experiments with succinate as substrate, on the other hand, indicated that under these conditions the respiration of the brain from a thiamin deficient pigeon was entirely normal. Therefore, it was believed that thiamin functioned in the lactic oxidase system of pigeon brain.

Two objections to this view soon became apparent. First, Birch and Mann (6) showed that the coenzyme for the oxidation of lactic acid did not contain thiamin. Then Meikeljohn (7), working in Peters' laboratory, found that the increased oxygen uptake caused by the addition of thiamin to polyneuritic pigeon brain respiring in the presence of lactate was not accompanied by an increased removal of lactic acid. Later pyruvic acid was employed as the substrate, and it was found that, contrary to an earlier observation (7), the addition of thiamin stimulated both the oxygen uptake and pyruvate removal (8). These observations were verified in Elvehjem's laboratory (9, 10, 11) and the studies were extended to other tissues such as liver, kidney and heart. It was found that similar effects could be observed with the kidney and heart, and that the respiration of liver from thiamin deficient animals was below normal, even when the complicating effect of inanition was eliminated by forced feeding. The conclusion was drawn that thiamin was essential in the metabolism of all the tissues studied (11), a view which is consistent with the universal distribution of the vitamin in animal tissues, and its indispensable role in carbohydrate metabolism.

In vitro studies are always subject to the criticism of artificiality, since they are performed under conditions which are far from physiological. The *in vivo* verification of such studies is necessary in order to establish the fundamental physiological significance of the findings of the *in vitro* work. This has been done in the case of thiamin, for it has been found that the lactic acid content of polyneuritic tissues is higher than normal (12, 13) especially after exercise. It has likewise been demonstrated that the pyruvate content of blood increases during polyneuritis and that there is a decrease after the administration of thiamin (14, 15, 47). Sherman and Elvehjem (16) have also shown that injected pyruvate is removed less rapidly by a chick suffering from polyneuritis than by a normal animal. The increased pyruvate content of blood has been mentioned as a cause of the bradycardia observable in thiamin deficiency. However, Lu (47) has shown that the bradycardia of polyneuritis is cured more rapidly than the pyruvate is lowered to a normal figure, when thiamin is administered to a deficient animal. The increased pyruvate is more properly the effect rather than the cause of deranged organ metabolism.

In 1932 Simola (17) investigated the coenzyme content of the tissues of rats suffering from a complicated vitamin B deficiency and concluded that the cozymase content was not reduced but that the cocarboxylase content was significantly lowered in this condition. The full significance of this work was not clear until five years later when

Lohmann and Schuster (18) found that cocarboxylase was the pyrophosphoric ester of thiamin. This compound was isolated from yeast. It functions there in association with a specific protein to catalyze the decarboxylation of pyruvic acid to yield acetaldehyde and carbon dioxide. It was shown by these workers that cocarboxylase as well as thiamin showed an *in vitro* catalytic effect in the oxidation of pyruvate by polyneuritic pigeon brain, and that both cocarboxylase and free thiamin were active in curing polyneuritis.

The discovery of the coenzyme nature of thiamin pyrophosphate has stimulated considerable work during the past two years. It is this work with which we wish mainly to concern ourselves in this paper. The work may conveniently be divided into studies made in an attempt to answer the following four questions.

1. What is the relative distribution of cocarboxylase and free thiamin in tissues and how is this distribution affected by polyneuritis?

2. Is cocarboxylase or free thiamin active in the various metabolic reactions?

3. What is the mechanism by which thiamin is converted to cocarboxylase?

4. Through what chemical reactions does pyruvic acid proceed in metabolism and how does cocarboxylase enter into these reactions?

With reference to the first question, the evidence is predominantly in favor of the view that thiamin is present in animal tissues and yeast, mainly in the form of cocarboxylase. Ochoa and Peters (19) have used an enzymatic method for the determination of these compounds and conclude that there is considerably more cocarboxylase than free thiamin in animal tissues. This has been confirmed by Westenbrink and Goudsmit (20), who have used a modification of the thiochrome method for the determination of both compounds. These workers, as well as Ochoa and Peters (19), have also found a reduction in the cocarboxylase content of tissues in thiamin deficiency, in confirmation of the early work of Simola (17). Hennessy and Cerecedo (21) agree that in animal tissues and yeast there is predominantly cocarboxylase. On the other hand, Tauber (22), Hennessy (21) and Lohmann (18) find that the vitamin appears to exist mainly in the free state in plant tissues.

The second question, regarding the relative metabolic activity of cocarboxylase and free thiamin, has been answered with animal tissues, bacteria and yeast. In the case of animal tissues, the observation that there was relatively little free thiamin present, strongly suggested that the ester was the active form. However, contrary to the original observation of Lohmann and Schuster (18), it was found that free thiamin was more active in restoring to normal the oxidative level of polyneuritic pigeon brain than was cocarboxylase (23, 24). This was true despite the fact that simultaneous experiments indicated that under the conditions of the catatorulin test, pigeon brain synthesized cocarboxylase from added thiamin. Direct proof of the activity of cocarboxylase rather than free thiamin in this reaction, and an explanation of the anomalous results in the catatorulin test with thiamin and cocarboxylase has recently been achieved by Banga, Ochoa and Peters (25). These workers have employed finely ground suspensions of brain tissue and have made preparations which show no catatorulin test with free thiamin, while a good effect is observed with cocarboxylase. The explanation for the relatively greater effect of thiamin than of cocarboxylase under the conditions of the ordinary catatorulin test appears to lie in the impermeability of the tissue *brei* to cocarboxylase, the effect of free thiamin being produced after it is phosphorylated *in situ*.

With bacteria, Lipmann (26) and Barron and Lyman (24) have found that cocarboxylase rather than free thiamin is active in the oxidation of pyruvic acid. Lipmann (26) has used acetone dried preparations of *B. Delbrückii* which, when washed free from cocarboxylase, fail to oxidize pyruvic acid. The addition of thiamin has no effect upon the oxidation, but the addition of cocarboxylase results in the establishment of an enzymatically active system. Similarly, Barron and Lyman found that thiamin was inactive and cocarboxylase active in the oxidation of pyruvic acid by gonococci and hemolytic streptococci. Neither of these organisms is able to phosphorylate the free vitamin. *Staphylococcus aureus*, which is able to perform the phosphorylation, is stimulated by the addition of either thiamin or cocarboxylase.

In the case of yeast, Lohmann and Schuster (18) showed that cocarboxylase and not free thiamin was active in the decarboxylation of pyruvic acid to form acetaldehyde and carbon dioxide. This has been confirmed by many workers (27, 28, 29, 30). Ochoa and Peters (19), however, find that while thiamin itself fails to catalyze the decarboxylation of pyruvic acid in the presence of a cocarboxylase free enzyme prepared from bakers' yeast, it nevertheless exerts a powerful augmentative effect upon the action of small amounts of cocarboxylase. This observation has been employed in the criticism of our published results upon the mechanism of cocarboxylase synthesis, since the authors feel that the increased carbon dioxide evolution noted in our experiments when thiamin was added to our system was due not to the synthesis of the added thiamin to cocarboxylase, but was rather due simply to the activation of cocarboxylase already present in our system by the added thiamin. We have, there-

fore, investigated this phenomenon in some detail.

Studies of this type are performed by washing yeast with 50 volumes of M/10 Na₂HPO₄ either at room temperature or at 30° C. The yeast preparation obtained in this manner contains no cocarboxylase and fails to decarboxylate pyruvic acid, but it may be reactivated by the addition of cocarboxylase and Mg or Mn. Such a preparation is called atiozymase. We have found that when one microgram of cocarboxylase is added to an atiozymase preparation from brewers' yeast in the presence of pyruvic acid and a metallic activator, there is rapid evolution of carbon dioxide. Typical results in the presence and absence of added cocarboxylase are shown in columns 1 and 2 of Table I. Similar results may be obtained with a preparation from bakers' yeast, but great quantitative differences exist, for the brewers' yeast atiozymase is very much more active than that from bakers' yeast. Further differences between the two types of yeast may be noted when an excess of free thiamin is added to a system containing a small amount of cocarboxylase. Column 3 shows that the bakers' yeast enzyme is greatly stimulated by the addition of the thiamin, but that the increase in the presence of the brewers' yeast enzyme is either absent or very small, depending apparently upon the age of the dried yeast from which the atiozymase is prepared. We are thus far unable to explain the differences between bakers' and brewers' yeast preparations with respect to this phenomenon, but we have acquired some interesting information which eliminates some of the possibilities. First, the failure to achieve activation with the brewers' yeast enzyme preparation is not due to thiamin originally present in the system, since assay of this preparation by the bacterial growth method of West and Wilson (31)

indicates that there is less than 0.5 micrograms of thiamin or cocarboxylase present per gram of enzyme. Second, the activation of the bakers' yeast system by free thiamin cannot be due to synthesis of cocarboxylase from the thiamin added, since it occurs not only in the presence of iodacetic acid and sodium fluoride which we have shown to inhibit synthesis, but also in the presence of phosphate free buffers at a level of $2-3 \times 10^{-4}$ M phosphate. Third, the enzyme from brewers' yeast is much more active than that from bakers' yeast, even when the latter has been activated by the addition of thiamin. Fourth, the interpretation of our results suggested by Ochoa and Peters on the basis of their experience with bakers' yeast cannot be applied to our synthetic experiments, since the degree of activation of cocarboxylase by thiamin is so small. Finally, the physiological significance of the activation by free thiamin may be questioned because there appears to be little free thiamin in yeast, and also because activation of this sort is inefficient. Thus, we have found that the rate of decarboxylation of pyruvic acid by bakers' yeast atiozymase is as great in the presence of 4 micrograms of cocarboxylase as it is in the presence of one microgram of cocarboxylase and 10 micrograms of free thiamin. Since the literature indicates that there is predominantly cocarboxylase in the yeast cell, and since cocarboxylase is much more efficient a catalyst than is free thiamin, it appears possible that this type of activation has little significance in vivo. We may summarize the answer to the second problem by stating that the evidence is strongly in favor of the view that both the oxidation of pyruvic acid by bacteria and animal tissues, and the simple decarboxylation of pyruvic acid by yeast are catalyzed by cocarboxylase, and that the

TABLE I

The Activation of Cocarboxylase by Thiamin in the Presence of Bakers' and Brewers' Yeast Atiozymase Preparations

(Cocarboxylase (CO) = 1γ, B₁ = 10γ, iodoacetate = 0.005 M, NaF = 0.004 M, Na pyruvate = 10 mg.)

No.	Yeast	Buffer	CO₂ (mm.³/hr.)				
			—	CO	CO + B₁	Iodoacetate CO + B₁	NaF CO + B₁
1	Bakers'	Phosphate	0	19.4	286	215	—
2	"	Maleate + phosphate	0	176	469	—	520
3	"	Succinate	5	27.7	232	—	—
4	Brewers'	Phosphate	18.1	598	667	604	—
5	"	Maleate	13.5	550	565	—	—
6	"	Phosphate	137.0	654	724	—	—

free vitamin has only a doubtful function other than that of a precursor for cocarboxylase.

If cocarboxylase is the enzymatically active form of the vitamin, we may ask how it is formed from free thiamin. It has been shown by Tauber (32), Euler and Vestin (33), Lohmann and Schuster (18), Peters (23), Silverman and Werkman (34), Barron and Lyman (24), and ourselves (29, 30) that cocarboxylase may be synthesized by animal tissues, yeast and certain bacteria. We have previously shown that cocarboxylase may be synthesized from free thiamin by an atiozymase preparation from brewers' yeast, provided that hexosediphosphate and certain thermostable factors present in a boiled tissue extract are added. Synthesis in this manner was completely blocked by 0.003 M sodium iodoacetate, but was relatively unaffected by 0.04 M sodium fluoride. In order to further describe the mechanism of the synthesis we have attempted the identification of the essential constituents of the boiled tissue extracts. We have found that synthesis of cocarboxylase from free thiamin may be readily achieved if cozymase and acetaldehyde are substituted for the boiled tissue extract (Table II).

Using these materials we have found that a system containing atiozymase, hexosediphosphate, acetaldehyde, magnesium, manganese, cozymase and free thiamin will decarboxylate pyruvic acid. Omission of the thiamin or of the cozymase or hexosediphosphate, causes failure of this reaction. It may be seen that the synthetic reaction is inhibited completely by sodium iodoacetate, and it is inhibited to the extent of about 50 per cent by the addition of sodium fluoride. It may also be pointed out that in contrast to our early experiments with boiled tissue extracts, small amounts of acetaldehyde are necessary in order to achieve synthesis in the presence of cozymase. This is probably due to the fact that the boiled tissue extract contains some cocarboxylase which will decarboxylate the pyruvate present in the reaction vessel to furnish the acetaldehyde necessary for the further synthesis of cocarboxylase.

The inhibitory effect of iodoacetate, as well as the requirement for cozymase, hexosediphosphate, and acetaldehyde upon the synthesis of cocarboxylase makes it appear that the synthesis occurs as a result of the dismutation between triosephosphate and acetaldehyde, coupled with the esterification of inorganic phosphate as demonstrated in the synthesis of adenosine triphosphate by Meyerhof (35, 36, 37) and Needham (38). This reaction is completely inhibited by iodoacetate, but it is not affected by fluoride (36, 37, 39). The inhibitory effect of sodium fluoride in our experiments could readily be explained if it were true that the phosphoglyceric acid formed as a result of the dismutation reaction could be transformed to phosphopyruvic acid which might also be capable of phosphorylating thiamin. Under these conditions, sodium fluoride, which blocks the conversion of phosphoglyceric acid to phosphopyruvic acid (39, 40, 41) should also block the synthesis of cocarboxylase.

In order to check this point we have performed synthetic experiments using phosphoglyceric acid instead of hexosediphosphate and acetaldehyde. Table III shows that under these conditions synthesis may also be accomplished. But here the synthesis is completely blocked by sodium fluoride, while it is little affected by iodoacetate. It may also be noted that under these conditions cozymase is not required for the synthesis. This is in accord with the observation that the dephosphorylation of phosphopyruvic acid does not require cozymase, but only magnesium and adenylic acid.

The experiments to which we have referred indicate that two mechanisms are available for the synthesis of cocarboxylase. The first, involving the use of hexosediphosphate, cozymase and acetaldehyde may be isolated by the use of sodium

TABLE II

Synthesis of Cocarboxylase

Each vessel contained 100 mg. alkaline washed yeast, 100γ Mg, 100γ Mn, and 10 mg. Na pyruvate. In addition, the flasks contained 100γ thiamin, 10 mg. HDP, 1 mg. acetaldehyde, 50γ cozymase, 0.005 M iodoacetate, and 0.04 M NaF as indicated.

Thiamin	—	+	—	+	+	+	+	+
HDP	—	+	+	—	+	+	+	+
Acetaldehyde	—	+	+	+	—	+	+	+
Cozymase	—	+	+	+	+	—	+	+
Iodoacetate	—	—	—	—	—	—	+	—
NaF	—	—	—	—	—	—	—	+
CO_2 mm.³/hr.	33	1373	76	36	226	125	164	526

Each vessel contained 100 mg. alkaline washed yeast, 100γ Mg, 100γ Mn, and 10 mg. Na pyruvate. In addition, the flasks contained 100γ thiamin, 5 mg. phosphoglyceric acid, 50γ cozymase, 0.005 M iodoacetate and 0.04 M NaF as indicated.

Thiamin	—	+	+	+	+	+
PO₄ Glyc.	+	+	+	—	+	+
Cozymase	+	+	—	+	+	+
Iodoacetate	—	—	—	—	+	—
NaF	—	—	—	—	—	+
CO₂ mm.³/hr.	37	697	638	23	467	18

fluoride. Under these conditions the phosphoglyceric acid formed as the result of the dismutation of triosephosphate and acetaldehyde could not be transformed into phosphopyruvic acid. The other mechanism available for the synthesis may be isolated simply by the use of phosphoglyceric acid as described.

The resemblance of the mechanisms described for the synthesis of cocarboxylase to those described by Parnas, Ostern, Meyerhof, Needham and others for the synthesis of adenosinetriphosphate from adenylic acid in yeast or muscle extract is clear. In addition, it has been shown that the mechanisms for the phosphorylation of glucose by yeast, and glycogen and creatine by animal tissue extracts all involve adenylic acid or its phosphorylated derivatives as phosphate transferring agents. The question may therefore be raised as to whether the phosphorylation of thiamin involves the intervention of the adenylic acid system, or whether the thiamin is phosphorylated directly.

It will be apparent that in the experiments which we have cited adenylic acid was not added to the synthetic medium. It was essential, therefore, to determine whether or not adenylic acid was present in the atiozymase preparation. This was done by determining whether or not glucose could be fermented with the addition of only cozymase and cocarboxylase to the medium. The results of a typical experiment are shown in Fig. 1. It will be seen that in the presence of atiozymase, Mg, Mn, cocarboxylase and a small amount of hexosediphosphate and acetaldehyde in order to eliminate the lag period, glucose is readily fermented (curve 1). This may be contrasted with curve 2 in which the glucose has been omitted. The rate of synthesis of cocarboxylase under the same conditions, but in the presence of added pyruvate, is shown in curve 3. Similar experiments in which the fermentation of phosphoglyceric acid has been studied show that this com-

pound is also readily fermented by our atiozymase preparations in the presence of added cocarboxylase and metallic activators only.

The fermentation of glucose in the presence of cozymase, cocarboxylase, hexosediphosphate and acetaldehyde, and the fermentation of phosphoglyceric acid in the presence of only magnesium, manganese, and cocarboxylase, indicates that adenylic acid must be present in our atiozymase. This is contrary to the results obtained by Lohmann and Schuster (18) who, using the same criteria for its presence, found no adenylic acid in atiozymase preparations. The difference between these two results is probably explained by the preparation of the dried yeast. As stated previously, the conditions for washing out cocarboxylase vary considerably with the yeast employed and the method of drying it. This is probably also true for the adenylic acid present in the yeast.

The important question remaining, then, is whether or not cocarboxylase is synthesized through the intervention of the adenylic acid system, or whether it is phosphorylated directly, either by the esterification of inorganic phosphate or by the direct transfer of phosphate from phosphopyruvic acid.

In an effort to answer this question, we have attempted to modify the usual procedure for the preparation of atiozymase in order to remove the adenylic acid. Various procedures involving different degrees of washing, alternate washings with acid and alkaline phosphate, and different drying temperatures for the unwashed yeast have been attempted. In all of these, the troublesome factor
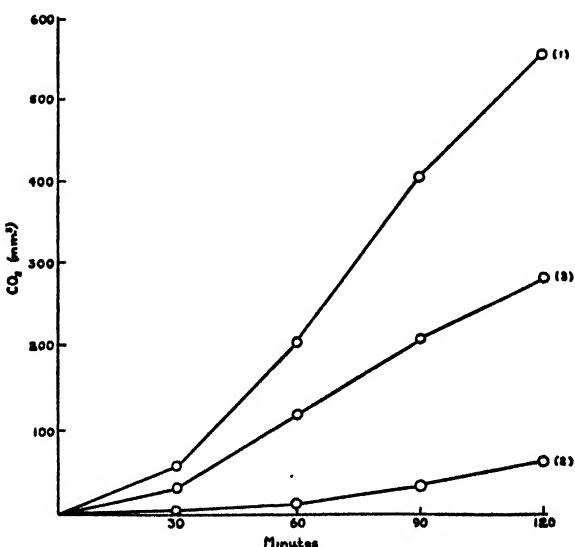
Fig. 1. Fermentation of glucose and synthesis of cocarboxylase in the absence of added adenylic acid. (1) Fermentation of glucose; cocarboxylase added. (2) Fermentation control; no glucose; cocarboxylase added. (3) Synthesis of cocarboxylase.

lies in the instability of atiozymase in the absence of cocarboxylase. Since under our conditions cocarboxylase is washed out more readily than adenylic acid, extensive washing for the removal of the latter compound tends to inactivate the atiozymase. Thus far, the most successful procedure for the removal of adenylic acid while still maintaining an active atiozymase appears to consist of two preliminary washes with acid phosphate followed by rapid drying of the yeast. The preparation is then washed with alkaline phosphate and water. Such a preparation still contains some adenylic acid, but a definite increase in the degree of cocarboxylase synthesis when adenylic acid is added may be noted. A typical experiment in which the effect of adenylic acid upon the synthesis of cocarboxylase by both the phosphoglyceric acid and the hexosediphosphate-acetaldehyde path is shown in Fig. 2. It will be seen that the addition of adenylic acid results in a greater synthesis of cocarboxylase by either path. These results are best explained on the basis that thiamin is phosphorylated by means of the phosphate transferring reactions of the Embden-Meyerhof scheme with the intervention of the adenylic acid system. It appears that adenylic acid is first phosphorylated and then transfers phosphorus to thiamin in a manner analogous to that of the phosphorylation of creatine in muscle. Further evidence for this view may be derived from the observation of Euler and Vestin (33) that cocarboxylase may be synthesized in whole dried yeast, provided that adenosinetriphosphate or hexosediphosphate is added. However, conclusive proof of this reaction must await experimentation with adenosinetriphosphate in an isolated enzyme system.

We would like at this point to refer briefly to the quantitative aspects of the synthesis which we have achieved. These experiments have been performed by incubating the synthesizing mixture for one hour, and then precipitating by boiling and centrifuging. The centrifugate is then assayed for cocarboxylase enzymatically in the presence of iodoacetate and fluoride so that further synthesis will be prevented. The carbon dioxide production under these conditions may be referred directly to a calibration curve obtained with pure cocarboxylase in the presence of these inhibitors. The results of a typical experiment are shown in Table IV. It may be seen that only small amounts of cocarboxylase are synthesized under our conditions, but the efficiency of the synthesis is quite high. Thus, while only 4.2 micrograms of cocarboxylase are synthesized even when 100 micrograms of thiamin are added, the addition of one microgram of thiamin results in the synthesis of 1.45 micrograms of cocarboxylase. The theoretical amount of cocarboxylase equivalent to one microgram of thiamin is 1.53.

The small amount of cocarboxylase synthesized suggests the possibility that only sufficient cocarboxylase will be formed to permit saturation of the atiozymase. We have performed many experiments with various levels of cozymase, hexosediphosphate, and phosphoglyceric acid, but have never achieved synthesis of more than 7 micrograms of cocarboxylase with 100 milligrams of atiozymase. This would be equivalent to placing back in the atiozymase preparation from 50-70 micrograms of cocarboxylase per gram of enzyme. Since fresh brewers' yeast commonly contains only about 100 micrograms of thiamin per gram, and since some loss of enzyme activity is to be expected as a result of washing and drying, it may be seen that a considerable portion of the cocarboxylase originally present in the yeast may be put back into the yeast by our procedure. The view that only sufficient cocarboxylase may be synthesized to saturate the yeast protein may also be the explanation of the failure of Kinnersley and Peters (46) to synthesize cocarboxylase with dried unwashed bakers' yeast.

Turning now to the fourth question, it will be apparent that the elucidation of the specific reactions in which cocarboxylase is enzymatically active is dependent upon the development of specific
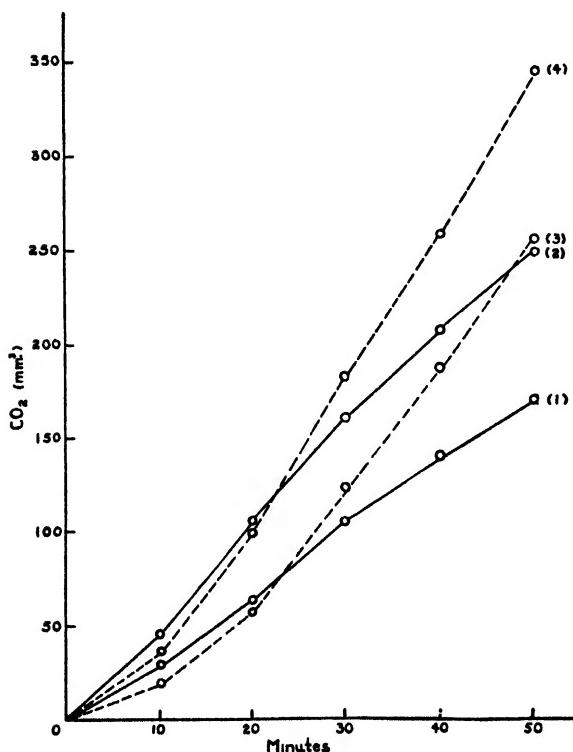
Fig. 2. Effect of adenylic acid addition on the synthesis of cocarboxylase. Solid line: phosphoglyceric acid. Broken line: HDP, acetaldehyde and cozymase. (1) and (3) No adenylic acid. (2) and (4) Adenylic acid added.

TABLE IV

Quantitative Determination of Synthesis of Cocarboxylase

Synthetic mixture—100 mg. atiozymase, 100γ Mg, 100γ Mn, 10 mg. HDP, 1 mg. acetaldehyde, 10 mg. pyruvate, and 16γ cozymase. Thiamin added as indicated.

Assay—in the presence of iodoacetate and fluoride. CO_2 $(mm.^3/hr.)$ referred to a calibration curve with pure cocarboxylase, B_1, and inhibitors.

Thiamin added (micrograms)	Aliquot	CO_2 $(mm.^3/hr.)$	Cocarboxylase found (micrograms)	Per cent conversion
100	0.5	551	4.32	3.3
10	0.5	504	4.14	31
5	1.0	891	4.2	55
1	1.0	369	1.45	95
0	1.0	0	—	—

information with regard to the metabolic paths of pyruvic acid. This is difficult, since it appears that pyruvic acid is the most reactive intermediate in carbohydrate metabolism, and that it is a key compound in the direct oxidation of carbohydrate by muscle, and in the conversion of carbohydrate into protein and fat.

The role of cocarboxylase in the metabolism of pyruvic acid by dried yeast is quite simple, since such yeast is capable simply of decarboxylating pyruvic acid with the formation of acetaldehyde and carbon dioxide. In animal tissues and many microorganisms, however, this reaction apparently does not occur, for the formation of acetaldehyde in these cells has never been demonstrated. On the other hand, it has been found that when a tissue is incubated *in vitro* with pyruvic acid under varying conditions, a wide variety of compounds including acetic, lactic, acetoacetic, and succinic acids are formed.

Examination of the literature indicates that there are four main paths through which pyruvic acid may be oxidized. The simplest of these is the direct oxidative decarboxylation of pyruvic acid to acetic acid:

pyruvic acid $+ 1/2$ $O_2 \longrightarrow$ acetic acid $+ CO_2$.
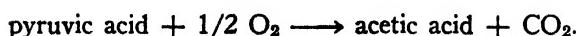
This reaction has been demonstrated to take place under aerobic conditions in *B. Delbrückii* by Lipmann (26) and in gonococci and hemolytic streptococci by Barron (24). In the case of these organisms, both workers have shown that cocarboxylase and not free thiamin is active. The same reaction has been shown to hold in the case of pigeon brain by Long (48). This reaction, according to this worker, accounts for the oxidation of at least 20 per cent of the pyruvate metabolized *in vitro*. Direct proof for the interaction of cocarboxylase in this reaction in animals is not available, but the fact that the addition of pure cocarboxylase to pigeon brain respiring under aerobic conditions in the presence of pyruvate increases the respiration, strongly suggests that cocarboxylase is a catalyst for this specific reaction.

Under anaerobic conditions Krebs (49) has demonstrated a reaction involving two ketonic acids, which results in the oxidative decarboxylation of one with the reduction of the other according to the following scheme:

$$RCOCOOH + R_1COCOOH =$$
$$RCOOH + CO_2 + R_1CH(OH)COOH$$
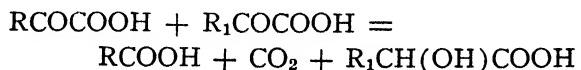
The interaction of two different ketonic acids under these experimental conditions is not necessary, for two molecules of the same acid may react. Thus,

2 pyruvic $= 1$ acetic $+ 1$ lactic $+ CO_2$

Krebs (50, 51) has demonstrated that this reaction occurs in animal tissues as well as in *Staphylococcus aureus*, and he is of the opinion that the oxygen uptake observed in the oxidation of pyruvic acid is due simply to the secondary oxidation of the lactic acid formed as a result of the dismutation. Weil-Malherbe (52) has confirmed the existence of this reaction in brain, and Barron (24) has demonstrated it in the metabolism of various microorganisms. These workers disagree with Krebs, however, since they feel that the

reaction is not the sole path for the oxidation of pyruvic acid. They believe instead that the strictly aerobic oxidation to acetic acid is quantitatively more important than the anaerobic dismutation. Regardless of the relative importance of these two reactions, we may state that cocarboxylase, and not the free vitamin B_1, has been demonstrated to be essential for the dismutation reaction in bacteria by Barron and Lyman. Whether cocarboxylase is required for the analogous reaction in animal tissues is not known.

The relation of cocarboxylase to the conversion of carbohydrate into fat is not so definite. The nutritional studies of McHenry (53) and Church and Whipple (54) indicate that there is a greater synthesis of body fat by rats in the presence of a ration containing adequate thiamin. Since Krebs (55) has shown that pyruvic acid and acetic acids seem to be the precursors of acetoacetic acid, and since cocarboxylase functions in the oxidation of pyruvic acid and in the formation of acetic acid, it is likely that cocarboxylase may play an important role in fat synthesis.

The final scheme which has been elaborated in an effort to elucidate the various paths through which pyruvic acid may be metabolized is that which has developed from Szent-Györgyi's (56) succinic acid cycle, and which has been termed by Krebs (57) the citric acid cycle (Fig. 3). It will readily be seen that in this cycle several oxidative decarboxylations occur. Thus the decarboxylation of oxalomesaconic acid to yield cis-aconitic is

oxidative, as is likewise the conversion of α-ketoglutaric acid to succinic. It is tempting, therefore, to postulate the necessity of thiamin in both of these reactions. This raises the question of whether the cocarboxylase system in animal tissues is specific for pyruvic acid, or whether cocarboxylase may also function in the oxidation of other α-ketonic acids. Neuberg (63) showed many years ago that yeast decarboxylated many α-keto acids containing at least one β-hydrogen atom. Long and Peters (62) have shown that cocarboxylase is essential in this system for the decarboxylation of α-ketobutyric and α-ketovaleric acids. They have further shown that α-ketobutyrate is readily oxidized by pigeon brain and that a catatorulin effect similar to that observed with pyruvate may be observed with this compound; α-ketovalerate is also oxidized by pigeon brain, but to a much less extent than pyruvate or α-ketobutyrate. The fact that compounds other than pyruvate appear to require cocarboxylase for oxidation leads to the possibility that the keto acids of the Krebs cycle also require cocarboxylase.

Direct evidence for the interaction of thiamin in this cycle is not at hand, but indirect evidence is available. Thus Simola (58) has found that the excretion of α-ketoglutaric acid in thiamin deficiency rises to a degree corresponding to that of pyruvic acid. This, of course, indicates that the metabolism of α-ketoglutaric acid is abnormal in thiamin deficiency. It has likewise been reported in a short note by Krebs (59) that the excretion
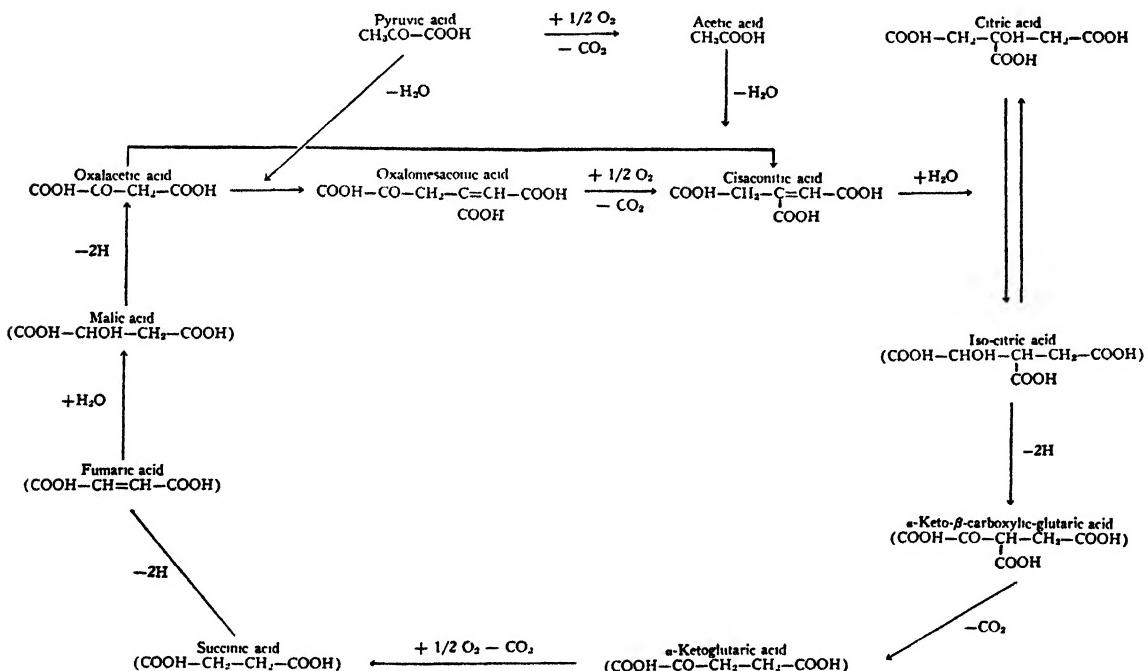
Fig. 3. The Citric Acid Cycle

of citric acid is abnormally high in thiamin deficiency. His results are not in harmony with our concept that thiamin is essential in the citric acid cycle, since according to this view the formation of citric acid from pyruvic acid should be abnormally low in the absence of thiamin. We have investigated the excretion of citric acid in thiamin deficiency in our own laboratory, and contrary to the results of Krebs, find that there is actually a small decrease in excretion of citric acid in polyneuritis.*

In view of this observation, the effect of thiamin injection upon the citric acid excretion of thiamin deficient rats has been studied with the results shown in Fig. 4.
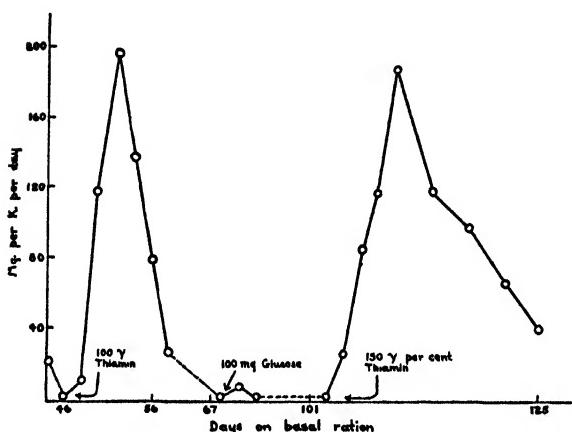
Fig. 4. Effect of thiamin on urinary citric acid excretion.

It may be seen that a single injection of 100 micrograms of thiamin into a rat results in a tremendous increase in citric acid excretion, and that this large excretion gradually returns to normal. Similar results have been obtained upon feeding thiamin. Administration of thiamin to normal animals produces no increase in citric acid excretion.

Orten and Smith (60) have shown that succinic acid injected into an animal results in a tremendous excretion of citric acid, and this has been confirmed by Krebs (61) and incorporated as evidence for the citric acid cycle. According to our view, the conversion of succinic acid to citric acid should also require the presence of thiamin, and we have therefore determined the effect of intraperitoneal injections of sodium succinate upon the excretion of citric acid by normal and thiamin deficient rats.

Starting with animals deficient, but exhibiting no symptoms, and injecting them with sodium succinate every other day, we have found that the

* These experiments have been performed in collaboration with Mr. H. A. Sober.

conversion of this compound to citric acid diminishes with increasing severity of polyneuritis. An alternative explanation for these results would involve the assumption that the rate of destruction of citric acid increases in thiamin deficiency. This is unlikely, however, since in a cycle of this sort it would be expected that the accumulation of an end product like a-ketoglutaric acid would if anything decrease the speed with which the citric acid is catabolized.

In answer to the fourth question, therefore, we may state that while the evidence is not complete, there is some reason for believing that cocarboxylase is essential for every transformation of pyruvic acid which involves oxidation. This includes the direct oxidation to acetic acid, the anaerobic dismutation, the conversion to acetoacetic acid, and the transformations involved in the Krebs citric acid cycle.

Other interesting work upon the mechanism of cocarboxylase action has been performed by Lipmann, but this will be reported by him later in this Symposium. We should like to mention but one observation which appears to be quite significant. He has found that the oxidation of pyruvic acid by bacteria requires not only cocarboxylase, but also flavin phosphate as a hydrogen carrier. If we may be rash enough to expect that fundamental reactions in the metabolism of one class of organisms may be transposed to other organisms, we find an interesting interrelation between three of the vitamins of the B complex. Nicotinic acid, as a constituent of the cozymase molecule, appears to be essential for the formation of cocarboxylase, and riboflavin for its action.

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DISCUSSION

Dr. Barker: Any role of cocarboxylase in the reactions concerning carbohydrate which enters into the Krebs cycle is hypothetical. Krebs himself has presented no evidence as to what carbohydrate material is involved in the citric acid cycle. He implicates pyruvate, but without any well established basis. I bring this up merely to suggest that whether or not you get an increased excretion of citric acid in polyneuritis may have no bearing on the Krebs cycle.

Dr. Lipton: It is true that Krebs has presented no evidence that pyruvate is the triose which combines with citric acid to ultimately yield citric acid. However, Simola has shown that the injection of pyruvate into rats produces a rise in citric acid excretion similar to that caused by the injection of succinate. Furthermore, while it is true that the explanation which I have offered in order to correlate our results with "the citric acid cycle" is hypothetical, the experimental results are quite conclusive, and they indicate that thiamin does influence the rate of citric acid excretion by the rat.

Dr. Barker: Since your polyneuritic animals were on a basal diet throughout the experiment, is there any possibility that an increased food intake caused by the generally improved physical condition of the animal following thiamin might account for the heightened citrate excretion?

Dr. Lipton: I do not think so, and can offer three lines of evidence against this possibility. First, the ration is highly purified and contains 76 p.c. sucrose and 18 p.c. casein; it is quite low in preformed citrate. Second, an injection of glucose into the polyneuritic rats fails to influence the citric acid excretion; forced feeding, then, has no effect. Finally, the citric acid excretion rises and then returns to a normal level when an animal is still gaining weight rapidly and is showing maximum food consumption.

Dr. Barker: Quantitatively, the excretion of citrate might depend upon the amount of precursors in the diet as well as of preformed citrate. If your diet is almost a synthetic one, it would rule that out more completely.

As to the significance of acetoacetic acid in the synthesis of the higher fatty acids, would you give us some further evidence? Acetoacetic acid is considered important in the breakdown, but I know of no evidence that it is concerned in the synthesis.

Dr. Lipton: It is unfortunately true that the intermediate steps in the conversion of carbohydrate to fat are still quite obscure, and that direct evidence in favor of acetoacetic acid as a precursor in the synthesis of higher fatty acids is almost completely lacking. Nevertheless, the conversion of acetoacetic acid to butyric acid has been demonstrated, and I think it plausible that an analogous mechanism is involved in the synthesis of the higher fatty acids.

Mr. Abrams: You stated, I believe, that the oxidation of pyruvic acid to acetic acid requires cocarboxylase. I wonder what role hydrogen

peroxide might play in that reaction. It is well known that hydrogen peroxide will very rapidly oxidize pyruvic acid to acetic acid, and when you use a dry yeast preparation, which undoubtedly contains yellow ferments of various sorts which will form peroxide from the oxygen, do you not think that the hydrogen peroxide might perhaps enter into the oxidation of pyruvic acid to acetic acid?

Dr. Lipton: A dry yeast preparation does not oxidize pyruvic acid. It simply decarboxylates it to form acetaldehyde. In the oxidation of pyruvic acid to acetic acid, all the available evidence favors the view that cocarboxylase is required for the reaction. Whether hydrogen peroxide plays a secondary role in this conversion is not known.

Dr. Lipmann: Do you think that your experi-ments with adenylic acid could give an explana-tion for the results of Albers, who maintains that under certain conditions decarboxylation can be enhanced by addition of adenylic acid? Then ac-cording to your experiments, adenylic acid would only catalyse the synthesis of thiamin pyrophos-phate but not decarboxylation.

Dr. Lipton: I think that the stimulatory effect of adenylic acid that Albers has noted may have two possible explanations. Either the adenylic acid has caused synthesis of cocarboxylase from free thiamin present in the system, or it acts by main-taining the glycolytic system intact. In this way it may aid in the removal of acetaldehyde formed by the decarboxylation of pyruvic acid. Acetalde-hyde exerts a definite inhibitory effect upon the rate of pyruvic acid breakdown.

THE RELATION BETWEEN THE "OXIDATION-REDUCTION POTENTIAL" AND THE OXYGEN CONSUMPTION RATE OF YEAST CELL SUSPENSIONS*

J. PERCY BAUMBERGER

I. INTRODUCTION

The purpose of the paper is to attempt to relate and to show what connection there exists between the potential impressed on a bare platinum electrode or on a dropping mercury electrode immersed in a yeast cell suspension[1] and the rate of oxygen consumption of the suspension.

The term "oxidation-reduction potential" is used loosely in this paper with the implication that an infinitely small quantity of an oxidation-reduction indicator of suitable E'_0 added to the suspension would be instantaneously reduced to such a degree that its electron pressure would correspond to the potential measured by an indifferent electrode in the suspension. This in no way implies that the cell suspension is in thermodynamic equilibrium. In other words, the numerous systems present are not necessarily in equilibrium with each other except in a dynamic sense. The ratios of oxidant to reductant of the different systems present would depart from the values calculable from their respective E'_0 and concentration because of great differences in reaction rates. The potential measured is represented by a steady state or dynamic equilibrium, the chief factors of which are the activation of hydrogen of the substrate by the dehydrogenase, and the combination of this hydrogen with oxygen; the potential is the measure of the activity of the mediators of this energy transfer. The problem is to discover what relation the potential of this steady state bears to the oxygen consumption rate. In other words, the problem is one of kinetics as well as potential.

II. RELATION OF RESPIRATORY RATE TO OXYGEN TENSION.

Warburg and Kubowitz (1) have made the most important studies of this subject. In 1929 they showed in a micrococcus, which they chose because it was such an extremely small organism, that diffusion within the organism itself would probably be a negligible factor, that the respiration was diminished to 50 per cent only when the oxygen pressure had been dropped from 1 atmosphere to 2×10^{-5} atmospheres at $10°$ C. Above this pressure the rate of oxygen consumption was practically independent of oxygen pressure. The independence of the oxygen consumption rate and the pressure of oxygen at the higher pressures was due, according to their theory, to the fact that the respiratory enzyme reacted so quickly with oxygen as to be fully oxidized even at very low oxygen pressures. Since all of the oxygen had to be combined through reaction with the respiratory ferment, this fact determined that the rate of oxygen consumption would be independent of the oxygen pressure. Tang (2), in 1933, reviewed the subject and listed a number of examples of relatively high critical pressures of oxygen; in other words, the pressure below which the rate of oxygen consumption diminished was, for instance, from 20 to 228 mm. oxygen for various processes. Kempner (3) has further investigated this subject and presented at this Symposium a number of cases in which the Q_{O_2} changes with small changes in the pressure of oxygen. Warburg and Kubowitz suggest that in cell metabolism with oxygen below the critical pressure, the oxygen consumption was diminished as a result of the relative decrease in the oxidized iron of the respiratory ferment.

This paper deals primarily with yeast cells. Cook (4) made a study of the relation of Q_{O_2} and pressure of oxygen in the yeast cells and found that below a pressure of 64 mm. oxygen there was a decrease in the Q_{O_2}, and when the pressure of oxygen had dropped to 11.5 mm. the Q_{O_2} had diminished to 60 per cent of its value in air.

III. PHYSIOLOGICAL ROLE OF OXYGEN

Calorimetric measurements throw some light on the physiological function of oxygen in yeast. The method used is described in detail by Winzler and Baumberger (5). The findings run quite parallel to the important respiratory experiments of Stier and Stannard (10, 11).

Fig. 1 shows the rate of heat production as it varies with time in a well washed yeast suspension (5). This heat is associated with endogenous metabolism. Fig. 2 shows that no heat is produced in the absence of oxygen in washed cells (5). As soon as oxygen is admitted heat is produced at an increased rate for a period of time and then falls off to the normal endogenous heat production rate. This exaggerated rate is the payment of an "oxygen debt", but in a different sense than is usually implied by this physiological term. It is the oxidation of material which is more available for oxidation and which has been produced during the anaerobic period inside the cell. The fact that no heat was produced ac-

[1] Fleischmann's Bakers Yeast, *Saccharomyces cerevisiae*.
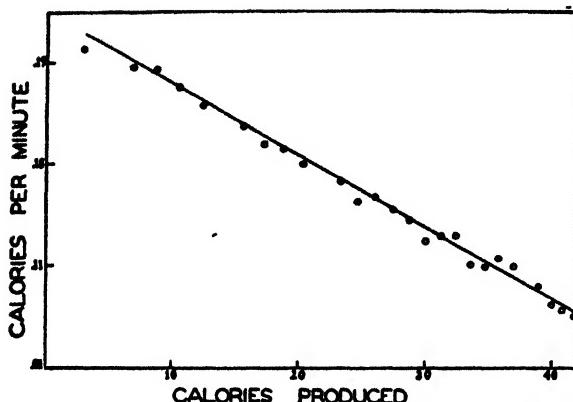
FIGURE 1.

companying that accumulation proves that it is not lactic acid, but may be sugar mobilized from glycogen. The point is that there is practically no energy liberated in the absence of oxygen by a cell dependent on its own stored products. However, there are sufficient stored products present to elicit a very vigorous heat production after the oxygen is admitted. In Fig. 3 is indicated the heat production of exogenous metabolism in the absence of oxygen taking place in the first portion of this curve (5). The amount of heat that was produced was accounted for by the formation of the end-products of fermentation, and also by a synthesis of a portion of those end-products into carbohydrate-like substances. When oxygen is admitted at a, there is a rapid oxygen consumption again.

A third point is that the growth of (beer) yeast [Windisch (6), Koch, Bengstsson and Hoffman (7)] does not continue in the absence of oxygen. Is this because no energy is liberated intracellularly and the cells cannot use the extracellular

FIGURE 2.

energy? Possibly certain essential compounds necessary in growth cannot be synthetized except in the presence of oxygen.

Another question is the relation of efficiency of utilization of energy and respiration. Efficiency (5) can be calculated as the ratio of the free energy used in synthesis to the free energy made available in a breakdown of the compounds. Seventy-three per cent of the glucose which disappears in exogenous metabolism in the presence of air is synthesized into carbohydrate compounds, and in the process there are only 7,320 calories stored as compared with 704,000 calories liberated in the complete oxidation of the glucose, 26 per cent of the glucose being oxidized. Aerobically for glucose

$$\text{efficiency} = 100 \times \frac{73.45 \times 7320}{26.55 \times 704,600} = 2.88 \text{ p.c.}$$
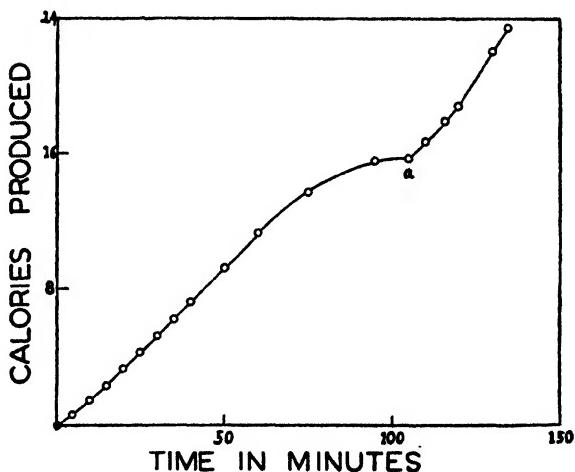
FIGURE 3.

Anaerobically with glucose the relations are 29.5 stored and 70.5 broken down to the end products of anaerobic metabolism. In that process 66,360 calories are liberated in the formation of fermentation products:

$$\text{efficiency} = 100 \times \frac{29.5 \times 7320}{70.5 \times 66,360} = 4.62 \text{ p.c.,}$$

so that the relative efficiency of the utilization of the energy which is liberated is twice as great as in the presence of air. However, in spite of the fact that the relative efficiency of the utilization of energy may be calculated to be greater, the heat produced may be even greater under anaerobic metabolism than in aerobic metabolism, therefore about ten times as much glucose is broken down anaerobically.
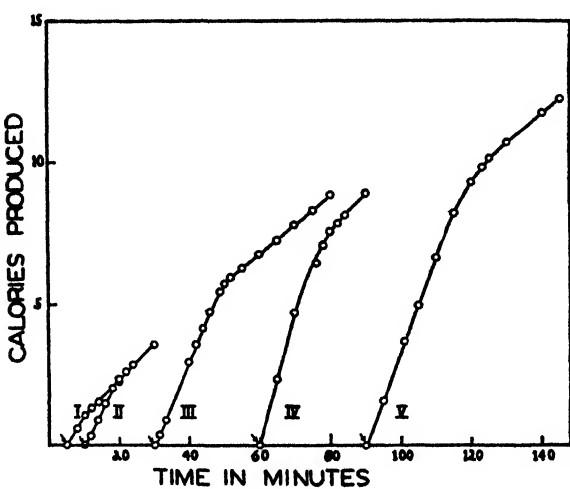
It would seem, then, that the principal function of oxygen would be to reduce the combustion of carbohydrate. Ball (8) has described a scheme of carbohydrate metabolism which may account for this change, and his scheme depends on the effect of oxygen. Oxygen, therefore, is the great fuel conserver, and that is probably its principal function. But the fact that the oxygen conserves fuel and at the same time yields a comparable amount of heat does not necessarily mean that this low grade energy is necessary or that the anaerobic energy is supplanted by the respiratory energy. The energy which is actually available for the major syntheses of the cell is certainly that which is associated with reversible processes. [Borsook (9) and Winzler and Baumberger (5)].

The oxidation of reduced cytochrome occurs appreciably only by oxygen and this is an irreversible process, therefore the energy liberated is necessarily degraded to heat. We can calculate that the highest expectancy of useful energy in carbohydrate oxidation must be less than that calculated as follows:

E'_0 cytochrome about 0.3;

$$\Delta F = - NEF = -6900$$

In oxidation of glucose 12 cytochrome molecules reduced = 82,700;

$$\text{efficiency} = 100 \times \frac{82,700}{704,600} = 12 \text{ p.c.}$$

Less than 12 p.c. of the energy of carbohydrate oxidation is associated with reversible reactions where it can conceivably be utilized for synthesis.

It therefore seems that the teleological argument of Ball is incorrect, when he says that "the action of oxygen in reducing the rate of carbohydrate destruction must, I think, be sought in the fact that the aerobic process by its complete combustion makes available the total energy of the carbohydrate molecule." The conservation of carbohydrate by oxygen is more likely a result of the change in the paths of metabolism which Ball has so well schematized, and a difference in the rate of hydrogen transfer over these different paths. Such possibilities are discussed further on.

IV. The Determination of Oxygen and Q_{O_2} by Means of the Dropping Mercury Electrode.

A. The Determination of Oxygen in Solution.

This method is suitable for the determination of gaseous or dissolved oxygen and has an accuracy comparable to other methods of analysis. It is based, in general, on the polarographic methods of Heyrovský (12) and in particuler, on the work of Vitek (13) in the determination of oxygen. The method here described is a simplification through the use of a single potential, suitable for oxygen determination. The method has been used since 1935 in a variety of ways for the direct determination of oxygen tension in the presence of cells (14-18). Petering and Daniels (19) have described a very similar method which really differs only in that measurements are made at two potentials instead of at one.

The determination of oxygen by the polarographic method has been carefully studied, particularly by Vitek. He has proved that the deflection of a galvanometer, in a circuit in which oxygen is reduced electrically at the dropping mercury electrode, is linear with oxygen concentration within the limits 0.5 p.c. to 100 p.c. with an accuracy of ± 2 p.c. of the oxygen present. This is confirmed by the work of Winzler and Baumberger (20). If the deflections of the galvanometer are photographed on a moving drum and the applied voltage indicated by abscissas, the type of polarogram (Fig. 4) obtained is like that used by Vitek.
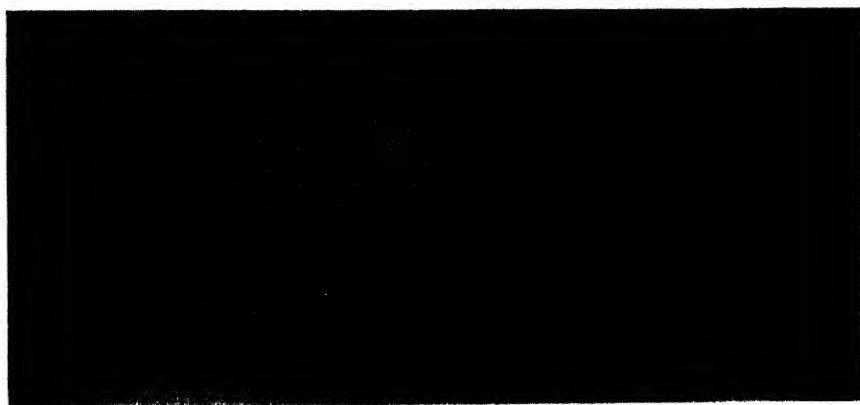
FIGURE 4.

In Fig. 4 the first abscissa is at zero applied voltage and therefore with a saturated calomel half cell in the circuit with $E_h = 0.250$, second abscissa $E_h = 0.150$, etc. The three curves are for phosphate buffer (pH 6.8) + caffeine saturated with (reading from top to bottom) oxygen, air, and nitrogen containing a trace of oxygen. On the first wave a plateau may be observed from E_h —0.150 to —0.350 and on the second wave at E_h —1.150 to —1.350. These regions give suitable potentials for single applied voltage oxygen determinations.

The first "wave", *i.e.*, the "limiting current" or "diffusion current" which is obtained when adsorption of the depolarizer is inhibited by a maxima suppressor (*e.g.*, caffeine) (Ilkovič, 22) is proportional to the concentration of the depolarizer (*e.g.* oxygen) and to its rate of diffusion and valence (Ilkovič, 23), the reaction probably being:

$$O_2 + 2H^+ + 2e \rightarrow H_2O_2$$

The second wave is also proportional to O_2 concentration and is probably due to the reaction:

$$O_2 + 2H^+ + 4e \rightarrow 2OH^-.$$
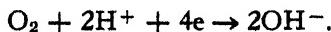
Since twice as many electrons are involved, the height will be twice as great.

Apparatus.

For a diagram of the dropping mercury electrode see p. 60. The E.M.F. chosen is 0.5 v. and this is obtained by tapping a 3,000 ohm resistance connecting the two poles of a dry cell. The potential applied to the drop is therefore —0.500 v. to the saturated calomel half-cell. The capillary is held by a rubber stopper firmly fixed in a clamp while the whole apparatus is rigidly held on a stand which, for very sensitive work, may be clamped to a vibration-free wall. The capillary is the most critical part of the apparatus. It can be readily made of thermometer tubing of fine bore which can easily be drawn down so that a slowly tapering lumen is obtained which will permit a drop of Hg every 1.5 to 6 seconds. The rubber pressure tubing should be repeatedly boiled in water and thoroughly dried before use. The mercury should be redistilled and dry. In the proper care of the electrode the following precaution must be observed: always have the mercury dropping from the electrode unless the tip of the electrode is under pure distilled water in which case the leveling bulb may be lowered until the dropping stops. This means that before the electrode vessel, full of distilled water, is removed, the bulb must first be raised until dropping starts, or while any solution is in the electrode vessel the mercury must continue to drop. In practice the leveling bulb is raised in the morning and not lowered until the work is ended. The mercury which falls while the electrode vessel is removed is collected in a wide flat dish of water. It is wise to have a large tray under the whole apparatus for emergency purposes as mercury on the floor may be dangerous to health.

The tip of the capillary should be far enough (about 10-20 mm.) under the electrolyte in the electrode vessel so that diffusion of gases from the gas-liquid interface does not interfere with the local environment of the electrode. In addition to having suitable conductivity the electrolyte should be a buffer, *e.g.*, M/10 (pH 6.8) phosphate. To this should be added a suitable suppressor of maxima, *e.g.*, caffeine. The galvanometer may be of the enclosed scale type with a sensitivity of 1.8×10^{-8} amperes, and is kept critically damped.

The deflections of the galvanometer are shown as ordinates in Fig. 4. The small rhythmic fluctuations on each curve are the result of change in current accompanying change in surface area of the drop as it grows and falls off. If the galvanometer deflections are not photographed on a moving drum they may be observed and the maximum deviation, *i.e.*, the crest, taken as the reading.

The deflection obtained when no oxygen is present may be determined by adding platinized asbestos and passing hydrogen through the solution. In this way any residual oxygen will be combined.

The deflection (D_a) obtained when buffer (pH 6.8) plus caffeine is saturated (by shaking) with air containing 20.97 p.c. O_2, the deflection (D_0) when zero oxygen is present, and the deflection (D_x) with the unknown, are used in calculating the unknown percentage oxygen thus:

$$\frac{D_x - D_0}{D_a - D_0} \times 20.97 = X$$

Since other reducible depolarizers are taken care of by the substraction of D_0, the deflections thus corrected are proportional to oxygen concentration and measurement at two applied potentials, as used by Petering and Daniels, is not necessary.

Use of the method in open circuit calorimetry.

An accuracy of 0.003 p.c. is attained in the usual volumetric method employed in the analysis of O_2 in metabolic work. A similar accuracy can be obtained with the present method. In open circuit calorimetry where the air may change in oxygen content only by about 1 p.c. it is necessary to measure a high concentration of oxygen.

In such experiments a high accuracy is secured by making the deflection of the galvanometer $D_a - D_0 = 3,000$ mm. by a long light lever, keeping the rotation of the galvanometer coil within the angle where response is linear. By averaging ten successive deflections (after equilibrating the electrode vessel solution with gas and allowing the fluid to become stationary) readings to 0.5 mm. are possible. Thus an accuracy of about 0.004 p.c. is secured.

Effects of temperature.

Given equal temperature of all of the apparatus, no effect of temperature on the oxygen determination was found. This was surprising since the amount of oxygen in solution is markedly affected by temperature. However, as Ilkovič (23) has shown, the diffusion constant of a reducible compound affects the current at the d.m.e.[2] With an increase in temperature, therefore, the resulting increase in diffusion rate would be expected to compensate to some extent for the decrease in solubility. A series of experiments at 0°, 10°, 20°, and 30°, using the high sensitivity method, in which outside air was bubbled through the buffer (pH 6.8) in the electrode vessel at each temperature, gave no difference in the deflection of the galvanometer. It must be emphasized that this is true only when the buffer temperature in the electrode vessel is in equilibrium with the gas phase at the temperature studied. If a solution in equilibrium with air is warmed without agitation, the deflection is increased. This is undoubtedly due to the fact that supersaturation for that temperature has occurred.

Since the increase in deflection exactly compensates for the decrease in solution of O_2 this is a fine example of application of Exner's Law (the coefficient of solubility is proportional to diffusion rate of a gas).

[2] The abbreviation d.m.e. will be used from here on for "dropping mercury electrode."

B. Determination of Oxygen Consumption Rate of Yeast Cells.

The d.m.e. was used to detect Q_{O_2} of yeast (or other) cells. The procedure was to make up, in suitable buffer medium, a cell suspension that would consume all the oxygen in solution in about 1 to 10 minutes. (No maxima suppressor was added when biological materials were used, as proteins, etc. serve this purpose.) The suspension was saturated with air by shaking vigorously in the electrode vessel, which was then quickly attached to the stopper. A photographic record of the change in deflection of the galvanometer with time is shown in Fig. 5. The rather uniform curve at the left shows the deflection with buffer plus caffeine after shaking with air. This serves as a reference point for the starting level of the deflection with yeast. The current-time curve shows some initial irregularities which are due to turbulence of the suspension, but these rapidly subside and then a line may be drawn along the crest of the waves and extended out to cut a horizontal line drawn along the regular portion of the crest of the buffer curve. Where these lines intercept is *zero time*. After a time, the deflections cease changing, and then correspond to D_0 as determined in cell suspensions. When this point is reached all of the oxygen has been used up, and the distance between zero time and final time is proportional to the oxygen consumption time and may be converted to hours. The tangent of the angle of the slope with the base line is proportional to the oxygen consumption rate.

$$Q_{O_2} = \frac{a}{\text{Wt.} \times \text{hours}}$$

$a =$ Bunsen solubility coefficient with O_2 expressed in mm.^{3}/cc.

Wt. $=$ mg. dry weight of tissue per cc.
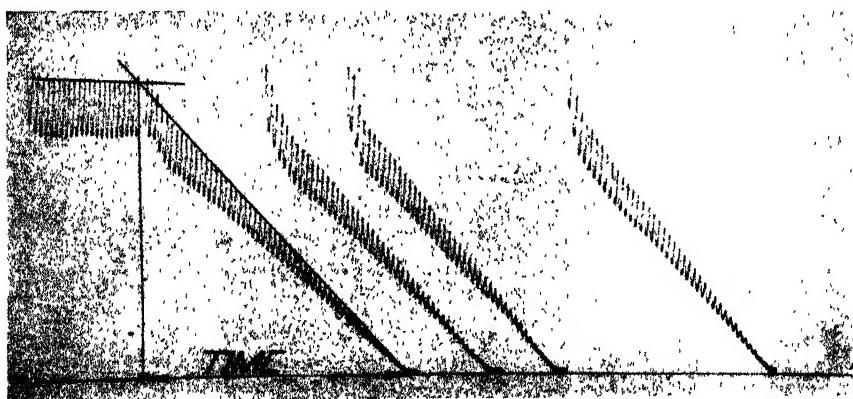
FIGURE 5.
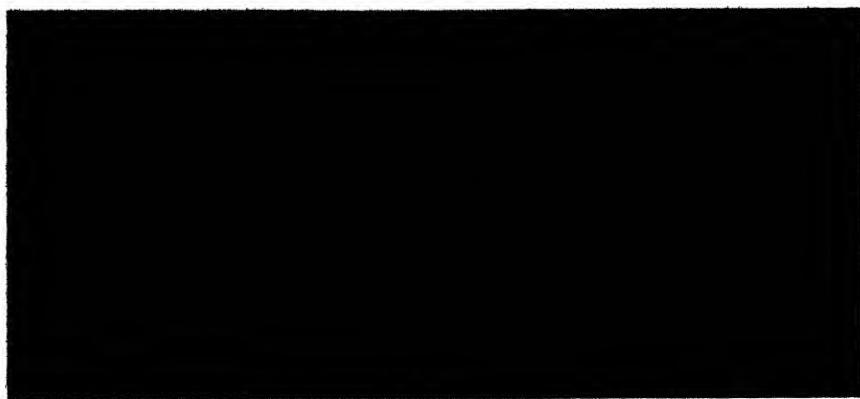
FIGURE 6.

If no photographic record is made, zero time may be closely estimated from a knowledge of the persistence of turbulence, and final time is obvious from uniformity of deflection. Time of oxygen consumption can therefore be determined without the photographic recording.

It might be expected that diffusion of oxygen would vitiate such experiments, but the following data (Table I and Fig. 6) show that when the electrode tip is 10-20 mm. below the surface, diffusion is too slow to play a role provided the suspension has a high enough oxygen consumption. In this series of experiments yeast suspensions in buffer of pH 6.8 plus 1 p.c. glucose were allowed to consume the oxygen in solution,

TABLE I.

Effect of Dilution on Determination of Oxygen Consumption Rate.

Curve	Concentrated yeast, p.c.	Observed angle		Tangent
A	1		65°	2.1445
B	0.5		50°	1.1918
C	0.16	(1)	27°	0.50953
C	0.16	(2)	25°	0.46631
D	0.033	(1)	5.7°	0.09981
D	0.033	(2)	5.5°	0.9629
D	0.333	(3)	5.5°	0.9629
D	0.033	(4)	5.5°	0.9629
D	0.033	(5)	5.5°	0.9629
D	0.033	(6)	6.5°	0.11394
D	0.033	(7)	4.0°	0.06993
D	0.033	(8)	3.0°	0.05241

* Based on measurements from Fig. 6.

and the galvanometer deflections were photographed on a revolving drum. The table shows that the rate of oxygen consumption does not diminish with dilution of cell suspensions until these are so dilute that several revolutions of the drum (at 15 min./revolution) are required for consumption of the oxygen in solution.

This method of determination of oxygen consumption rate was compared with the standard Warburg technique and found to agree favorably. The following example (Sept. 29, 1938) will serve to illustrate the point. The oxygen consumption rates of identically prepared 0.5 p.c. yeast in 1 p.c. glucose and M/10 phosphate buffer at pH 6.8 were determined at 25° C. on the same day and hour in four Warburg vessels and by graphic analysis of the photographic records of the d.m.e. technique. The manometric readings at 40 minutes in the four Warburg vessels after barometric corrections were 102, 110, 114, 102, and 126 with vessel constants of 0.406, 0.355, 0.363, 0.389, and 0.345, respectively, giving an average of 1.025 mm.^{3} oxygen consumed per minute. Graphic records of the current-time curve, similar to Fig. 5, show oxygen to have been consumed entirely in 92 mm. of record (where 16.5 mm. = 1 minute) and on repitition gave likewise 92 mm. With oxygen in solution (at 25° and saturation with air) equal to 0.0059 cc./cc. solution, the oxygen consumption rate works out as 1.075 mm.^{3}/min., i.e., the Warburg and the d.m.e. method agree in this typical instance within 5 per cent.

The question may arise as to whether or not the deflection $D_a - D_0$ is really due to oxygen or is partially the result of the accumulation or disappearance of other metabolities. In answer it can be said only that polarograms run on fully reduced yeast do not show any new substance to be present which is reducible on the d.m.e. at the voltage used for oxygen determination. If in

some tissue it should be the case, it would be demonstrable polarographically and could be corrected for.

Possible injury of Hg to the living cells has been considered by several authors (18, 19) but no injury has been observed. Repeated determination of oxygen consumption on the same yeast cell suspension by repeatedly shaking up with air and with the mercury which had dropped into the suspension in the determination, gave the current-time curves in Fig. 5. In these cases 5 and 10 minute intervals occurred between successive determinations.

V. RELATION OF THE E_h OF ADDED OXIDATION-REDUCTION INDICATORS TO THE RESPIRATORY RATE.

Barron and Hoffman (24) showed that the addition of those oxidation-reduction indicators which could be readily reduced by the cells increased the Q_{O_2}. Barron (25) has recently re-
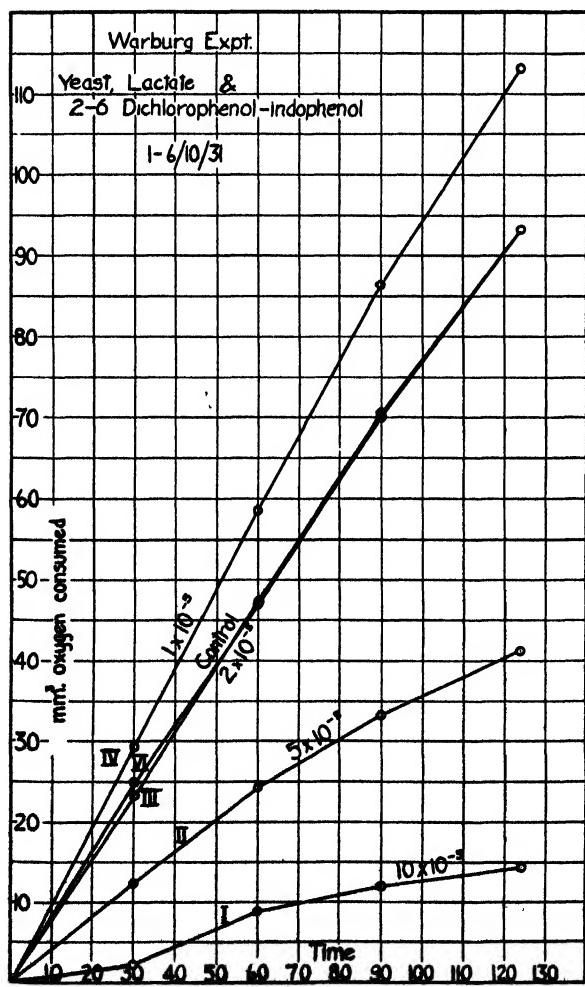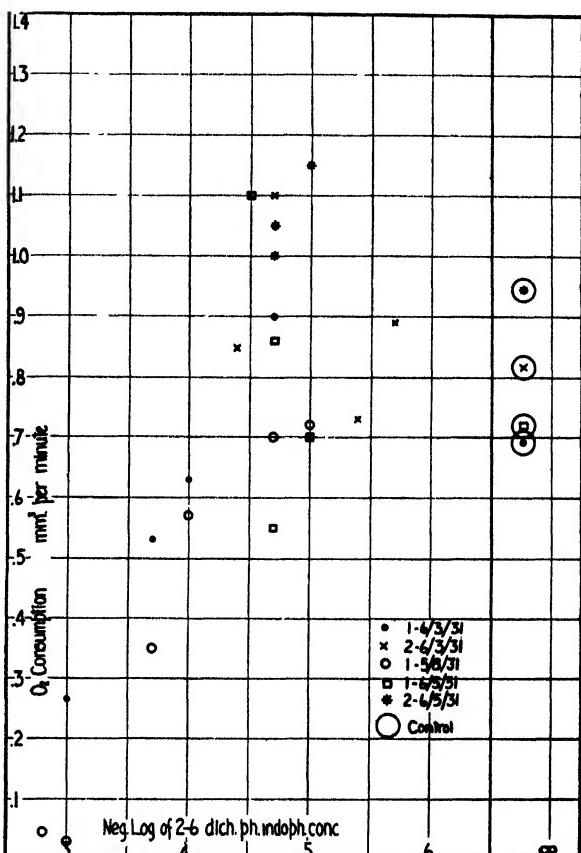
FIGURE 8.

viewed this subject. In 1931 the author made a number of such measurements and in Fig. 7 is shown a Warburg experiment with 1 cc. of 0.8 p.c. yeast in phosphate (pH 6.8), 0.1 M sodium lactate and 2-6 dichlorophenol-indophenol. The control is in the center and the same oxygen consumption is obtained with 2×10^{-5} M 2-6 dichlorophenol-indophenol. If the concentration of dye is increased there is a decrease in the Q_{O_2} of the yeast cell, but at a concentration of 1×10^{-5} it increases. Parallel experiments were run to determine the E_h of the cell suspension with oxygen bubbling and with the dye present. It was found (Fig. 8 and 9) that at E_h greater than 0.25 the Q_{O_2} diminished. This critical potential agrees with the E_h of the control cultures.

Burk (26) reviews the effect of oxidation-reduction indicators on the Meyerhof quotient. Since lactate is the substrate in the experiments described above and since no fermentation is involved, the effects on oxygen consumption must be direct and not through a change in Meyerhof ratio. Other experiments show that various oxidation-reduction indicators have a similar inhibitory action on Q_{O_2}, when in such concentra-
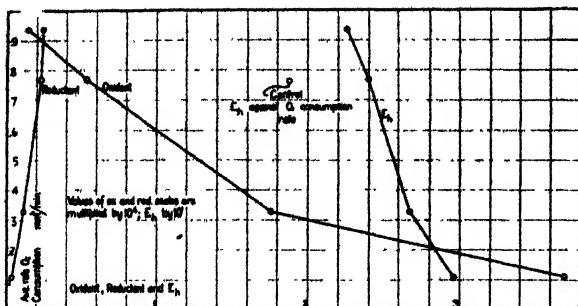
FIGURE 7.

FIGURE 9.

tion that in the presence of O_2, E_h is greater than 0.25. As in the case of the Meyerhof quotient E_h, and not the chemical constitution of the dye, is the critical factor.

VI. Relation of E_h of Cell Suspensions to Cytochrome Reduction.

A. E_h of Cell Suspensions.

The E_h of a cell suspension often goes through a typical course of change with age and aeration of a culture (27, 28, 29). This was shown by Clark (30), and many recent papers on this subject are reviewed by Hewitt (31). Kluyver and Hoogerheide (32) point out the excretion of oxidation-reduction systems into the medium may poise the electrode potential.

The potentials established by yeast cells depend upon the age, substrate, etc. Starch-free yeast suspended in buffer for 24 hours at 20° - 25° C. gave quite uniform results.

The technique employed (27) was as follows: The oxygen was bubbled through the suspension until the E_h remained constant for several minutes. This most positive E_h was called the "oxidation base". After this point had been established, the oxygen was shut off and the suspension was either permitted to stand or nitrogen was bubbled through. The appearance of the cytochrome bands was noted by the use of the hand spectroscope and the E_h at this point was recorded. The potential was followed until the most negative
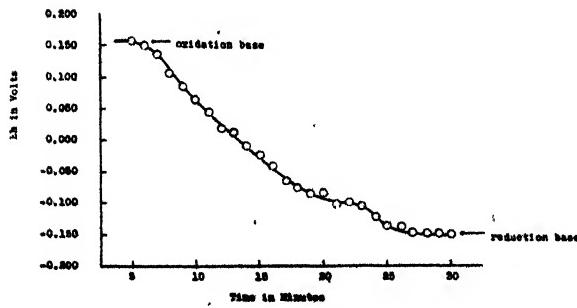
E_h was reached. This point was called the "reduction base". The experiments were run at 20° ± 3° except where otherwise stated.

Washed yeast cells give quite a different oxidation-reduction curve. The average value for the oxidation base of the 24 hour old suspension is 0.150 (see Fig. 10) and for the fresh suspensions 0.309. Similarly the cytochrome-c bands appear in their respective suspensions at 0.123 and 0.305. These differences disappear if the fresh yeast suspension is incubated at 33° for 4 hours or more, or if glucose is added up to 1 p.c., or if methylene blue or 1-naphthol-2 sulfonate-indophenol is added. These agents all lead to a greater electrode potential stabilizing power in the suspension. In the case of the stabilization by incubation the effect can be counteracted by the removal of the external milieu by centrifugation and the resuspension of the yeast in fresh buffer. Return of the yeast to the original suspension medium partially reestablishes the low oxidation base if the restitution is made within an hour, but fails if further delayed. It would appear as though some labile substance is liberated from the cells.

If a cell suspension which has been reduced for some time is reoxidized by bubbling air through it continuously (Fig. 11) the E_h starts
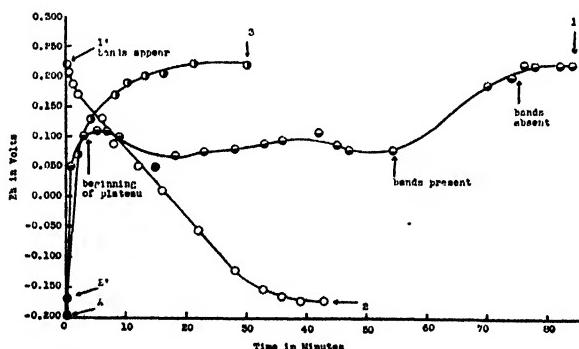
FIGURE 11.

at A and goes through a definite course to 1, showing a plateau with the bands of reduced cytochrome present. After a period of an hour some substance which has been formed in the absence of oxygen has finally been fully oxidized and the potential is then able to rise and the bands disappear.

When the same suspension is immediately reduced by its own anaerobic metabolism, E_h follows the course 1'-2, then reoxidizing, the E_h rises over course 2'-3 up to the oxidation base without any delay. Apparently whenever the formation of material which is slowly oxidizable is limited by short anaerobiosis, rapid return to the oxidation base can be obtained. This slowly oxidizable

FIGURE 10.

component of cell suspensions will be referred to again later on.

Clark (30), Michaelis and Flexner (33) and others have shown that bare platinum electrodes may act as oxygen electrodes giving fairly definite potentials varying with the oxygen pressure. It would appear that the fresh washed yeast suspensions do not affect the electrode sufficiently to overcome this oxygen electrode effect, but yeast suspensions will finally overcome the oxygen effect on the electrode when they have poured out sufficient metabolites into the external milieu. This occurs more rapidly if substrate or a highly reversible oxidation-reduction system like methylene blue is present. It is unlikely that the added substrate needs to penetrate into the cell in order to be modified, as Quastel and Whetham (34) have calculated that methylene blue is reduced at the cell surface.

The foregoing procedures make available a definitely reproducible preparation (Fig. 10) and in the work that follows weight is given only to potentials obtained from suspensions 24 hours old, or after incubation, or after addition of substrate or dyes.

Viability tests with 0.05 p.c. methylene blue in buffer of pH 6.8 showed no appreciable mortality in the various suspensions used. Cell counts and budding estimates made with the hemocytometer, and oxygen consumption determinations with added glucose by the Warburg method also showed negligible differences.

B. The Relative Catalase Activity of Fresh and Incubated Suspensions.

The probability that the high potential characteristic of fresh yeast suspensions is due to the accumulation of H_2O_2 (Hewitt, 35), because of a lower activity of catalase in the fresh suspensions as compared with incubated or 24 hour old suspensions, was tested as follows: H_2O_2 was emptied from the side arm of Warburg vessels into the yeast suspension and the oxygen liberated was measured. The results are given in Table II, and show that little, if any, difference exists between the fresh and incubated yeast. The rate of H_2O_2 decomposition was also determined by the graphic method of Bass-Becking and Hampton (36), and no difference in the rate of decomposition was noted. Furthermore, the oxygen consumption of the yeast as determined in the Warburg vessel is only a small fraction of the rate of H_2O_2 decomposition, so that it seems unlikely that this factor can account for the high potential of fresh yeast cultures.

C. The Significance of the Oxidation Base.

As previously stated, the "oxidation base" is defined as the highest level to which the E_h rises

TABLE II

Catalase Activity of Yeast

Yeast	Pressure of O_2 liberated from H_2O_2 in atm. $\times 10^{-4}$ in successive 2 minute intervals			
Expt. 7/23/34				
Freshly suspended	91	55	58	48
	84	47	52	43
Incubated 22 hr.	62	55	44	25
at 37° C.	62	50	47	27
Expt. 7/26/34				
Freshly suspended	56	77	66	42
	53	63	59	44
Incubated 22 hr.	66	71	69	35
at 37° C.	82	69	69	40

when air is bubbled through a 24 hour old yeast suspension. This level represents the balance of the forces of oxidation and reduction in the cell and its environment. The rates of activation of the hydrogen of the substrate by the dehydrogenases, and of oxygen by indophenol oxidase are involved, together with the rate of oxygen diffusion, autoxidation of cytochrome-b and of various mediators. Coupled oxidation-reduction systems (37) as well as reversible systems will come into equilibrium with, or rather will react upon, each other to establish an electron pressure interpretable as an E_h. From Fig. 10 it can be seen that the oxidation base is 0.160 ± 0.010 volts. The fact that the oxidation base does not rise more than 0.04 volts above the E'_0 of the cytochrome is strong indication that oxygen must be utilized largely, if not entirely, through combination with the hydrogen of reduced cytochrome which, of course, is in line with the modern theories of biological oxidation.

D. The Significance of the Reduction Base.

Similarly, the reduction base is determined by bubbling nitrogen or allowing yeast to stand until a constant low E_h is obtained. This E_h is a function of the pressure at which hydrogen can be liberated from the yeast cell substrates, etc. Unlike B. coli studied by Stephenson (38), S. cerevisiae cannot liberate hydrogen at one or more atmospheres. Consequently, the reduction base is considerably above the potential of the hydrogen electrode at the pH of the external milieu, or even

at the pH postulated for the cell interior. The reduction base actually found is —0.150, as shown in Fig. 10. Unlike the oxidation base, the reduction base does not represent the resultant of entirely opposing processes but, rather, the limit to which certain processes may proceed in one direction. Clark (30) and Wurmser (39) have discussed these questions at some length.

E. The E_h at which the Bands of Reduced Cytochrome Appear and Disappear.

Simultaneous observation of the appearance of the bands of reduced cytochrome (Keilin, 40) and the E_h in ten well poised yeast suspensions gave values of E_h from 0.150 to 0.110 with an average at 0.125, a result agreeing very well with $E'_0 = 0.123$ for cytochrome extract determined by Green (41). Beck and Robbins (42) likewise found agreement using the colorimetric method of determining E_h.

The possibility indicated previously that partially reduced cytochrome stablizes the potential of the system is given further support by the fact that the E_h of appearance or disappearance of the absorption spectrum of cytochrome-c always falls on the steepest portion of the E_h-time curve for any suspension. This fact can be readily seen in Fig. 10, 14, and 15.

The difference between the potential of band appearance and oxidation base averages 0.036 for normal conditions. It is tempting to speculate that when cytochrome is almost completely oxidized, the transfer of hydrogen to oxygen can no longer occur at the normal rate. Dehydrogenation processes then lower the potential again until the two processes balance each other.

F. The Spectrophotometric Determination of the Degree of Reduction of Cytochrome at Different E_h Values.

The direct observation of the degree of reduction of cytochrome is very difficult, if not impossible, because of the dilution in which it occurs in cells. Therefore a photometric method was necessary, and here the choice lay between spectrophotographic methods and photoelectric methods. Grateful acknowledgement is made to Dr. P. Leighton, Dr. J. H. C. Smith, and Dr. P. Kirkpatrick for helpful advice. The requirements of this work, however, are quite specific, and great credit is due Mr. R. K. Skow of this laboratory for the construction of the photoelectric amplifier finally used in this spectrophotometric work.

The apparatus may be briefly described as follows. The light source is a 32 or a 50 candle power Mazda headlight lamp, with heavy copper leads, operated on two 6 volt storage batteries in parallel. The lamp is kept cool by a fan. It is focused on the slit of the spectroscope with a 0.3 mm. aperture. This aperture was chosen because with it two yellow lines in the emission spectrum of the mercury arc, which are 20 A apart, appeared to fuse. Therefore, removal of the eye piece and replacement with a 0.3 mm. slit permits only a band of light of 20 A range of wavelengths to emerge. This is, then, the minimal unit of light selectivity of this apparatus. This band of light emerged from the slit in a black metal box and falls directly on the absorption vessel containing the yeast suspensions. Light transmitted falls on an RCA 868 gas filled photoelectric cell, sealed into a hole through a second compartment of the metal box. The advantage of this arrangement is that light entering the absorption vessel, though dispersed by the yeast cells, falls largely on the photoelectric cell, since the area of the latter is very much greater than the cross section of the entering light beam.

The second compartment of the metal box contains not only the photoelectric cell, but also an FP 54 pliotron amplifying tube, which is in a DuBridge and Brown (43) circuit with a 1.5×10^9 ohms White Dental Resistor on the grid. In this circuit the photoelectric current changes the grid bias and therefore the plate current, and the latter operates a H. S. type L. & N. galvanometer of a sensitivity of 1×10^{-9} amperes with a long light lever. The sensitivity of the apparatus is adjustable by a series of shunt resistances. The position of the zero of the galvanometer light beam is controlled by an opposing galvanometer circuit. The whole apparatus is shielded with the necessary care. The FP is run on storage batteries and the photoelectric cell on B batteries.

The yeast suspension through which the light passes is contained in a parallel sided glass vessel having a gas inlet and outlet, platinum electrode, and salt bridge. The salt bridge connects with a calomel half cell for reference.

Yeast is kept agitated by a stream of gas at constant line pressure, as determined by a water manometer. The composition of the gas may be nitrogen, air, or mixtures of the two. An arrangement of manometers permits fairly accurate reproduction of the gas mixture. Constant E_h levels are obtained by persistant bubbling of gas of a suitably low oxygen pressure. The transmission of light of known wavelength can then be determined at the same time as the E_h.

The spectrometer was calibrated against the emission spectrum of a mercury arc lamp. The deflection of the galvanometer is almost a direct linear function of the wavelength. This is the result of the spectral sensitivity of the photoelectric cell, the intensities in the emission spectrum of the light source, etc.

The apparatus is arranged so that the vessel containing the yeast suspension can be replaced

by a dye solution which permits determination of any variation in amplification so that subsequent galvanometer deflections may be corrected.

Fig. 12 shows three curves of the galvanometer deflection, *i.e.,* light transmitted for different wavelengths. The curve marked with open circles is the relative transmission when air was bubbling through the suspension and the E_h was in the neighborhood of 0.300. The curve marked with circles solid in the lower half was obtained at E_h 0.140, and the third curve, marked with circles with right hand half solid, was obtained at E_h 0.070.
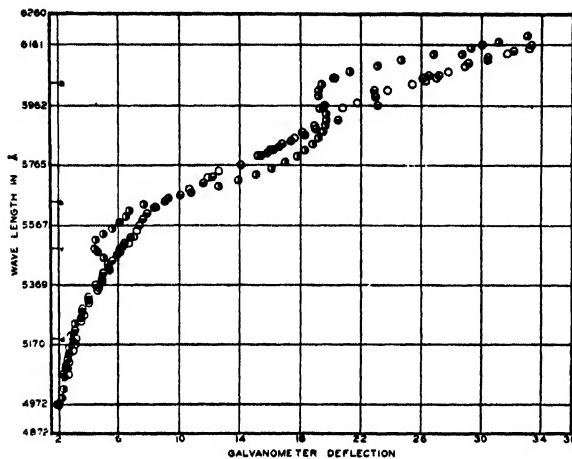
FIGURE 12.

In the interpretation of this data, the general slope of the curve is of less importance than the degree to which the curves differ from one another, as the method is a differential one. With this in mind, it can be seen that for certain wavelengths there is perfect agreement between all three curves. Throughout a still greater spectral region, there is perfect agreement between the curves at 0.300 and 0.140. In fact these two appear to differ appreciably only in the region of 5960 A and 6035 A. Here a decided deviation indicating greater light absorption occurs at E_h 0.140. This may indicate that the cytochrome-a is partially reduced at E_h 0.140, because the center of the absorption band of cytochrome-a occurs at 6035 A (as indicated on the left margin of Fig. 12) as discovered by Keilin (40). Furthermore, it can be seen that in this region absorption increases on the curve for E_h 0.070.

Therefore it would seem reasonable to assume that cytochrome-a is partially reduced at E_h 0.140 and fully reduced at E_h 0.070. However, close examination shows that a deviation of equal proportion to the total occurs in the region of the center of each of the absorption bands when the potential drops to 0.140. Therefore the E'_o of

cytochrome-a does not appear to be greater than that for -b or -c. Further evidence to this effect is presented farther on.

Cytochrome-c shows no absorption at E_h 0.300 and very little at E_h 0.140, but absorbs fully at E_h 0.070, therefore its E'_o must lie definitely below E_h 0.140. The cytochrome-b band shows up as a jog on the cytochrome-c absorption curve. It also is very slight at E_h 0.140, but strong at 0.070.

A great difference in light transmission occurs between the curve for E_h 0.070 and the other two curves (which are identical with each other) in the region 5660 to 5880 A. Here greater light transmission occurs as the E_h drops to 0.070, but no difference occurs between 0.300 and 0.140. This region is approximately the same as the region of absorption bands of oxidized cytochrome-c (Theorell, 44).

A calculation of E'_o of cytochrome-c was made from the data shown in Table III. Lambert-Beer's Law states that log $I_0/I = Kcd$ when:

$I_0 =$ light intensity with zero absorption

$I =$ light intensity after absorption

$K =$ extinction constant

$c =$ concentration of absorbing compound

$d =$ depth of the solution.

Since K and d are constant in any one experiment and I and I_0 are proportional to the galvanometer deflections, c can be calculated and is equal to the per cent of reduction of cytochrome-c from the above formula and from the formula:

$$E_h - E'_o = \frac{RT}{nF} \ln \frac{(Ox)}{(Red)}$$

where $n = 1$ according to Green.

The average E'_o calculated from this experiment agrees perfectly with Green's determination, but is about 0.13 volts negative to the values given by Coolidge (45 and 46), Wurmser and Filitti-Wurmser (47), Ball (48) and Stotz *et al.* (49).

The spectrophotometric method used was further improved upon in 1935 by (a) driving the prism of the spectrometer with a synchronous motor, (b) photographing, on a drum turning at 1000 seconds per revolution, the deflection of the galvanometer, (c) arranging a signal system to flash an absicca on the record at every 100 A, and (d) using a Corning 428 prism filter to obtain more suitable light energy distribution. A calibration curve using a neon lamp as source is shown in Fig. 13.

Furthermore in place of the null point method

TABLE III

Obs. E_h	Obs. I	Log (I_o/I)	Calc. p.c. red. cytochrome	Calc p.c. ox. cytochrome	Calc. E_h—E'_o	Calc. E'_o
0.295	16	0	0	100		
0.056	12.6	0.10346	100	0		
0.086	13	0.08991	88	12	—0.026	0.112
0.105	13.1	0.08707	84	16	—0.022	0.127
0.109	13.3	0.07027	67.8	32.2	—0.009	0.118
0.118	13.3	0.07027	67.8	32.2	—0.009	0.127
0.129	14.3	0.04922	47.5	52.5	0.001	0.128
0.147	15.8	0.00518	4.8	95.2	0.038	0.109
0.192	15.9	0.00260	2.5	97.5	0.047	0.145
					Average E'_o	0.1235

Note: $I_0 = 16$.

of E_h determination, a vacuum tube galvanometer was substituted. The position to which the galvanometer light beam deflected was then an indication of E_h. Fig. 14 is a photograph of the simultaneous deflection of the vacuum tube galvanometer (Enclosed scale L. & N., 0.019 mμ amp./5 mm., CDR 12,600, Res. 1105, Period 3 seconds) and the photometric galvanometer (H. S., L. & N., 0.0004 mμ amp./0.8 mm., CDR 10,500, Res. 530, Period 6.3 seconds). Such a record was obtained when λ was kept constant at 5490 A (the center of a reduced cytochrome-c absorption band) and when a yeast cell suspension became reduced on stopping bubbling of O_2. The record proceeds from right to left and simultaneous instants of time are points vertical to each other on the two curves. The E_h on the platinum electrode changes toward negative values and at the same time the reduction of cytochrome occurs with a very sudden increase in light absorption. E_h continues to decrease after all the cytochrome is fully reduced. On readmission of oxygen the cytochrome is rapidly reoxidized and the E_h changes to positive values less rapidly. A similar curve (Fig. 15) of E_h only, but with the observed appearance (with hand spectroscope) of the cytochrome-c band flashed on, shows that the E_h changes most sharply at the region where cytochrome is reduced. This was mentioned also in connection with Fig. 10 and is not an artifact, but is definitely reproducible.

In order to avoid lag which is characteristic of platinum electrodes in poorly poised systems the d.m.e. was used to follow the E_h changes (29). Müller and Baumberger (50, 51) described the use of this electrode with various reversible systems and for the determination of apparent oxidation-reduction potential (52). Here, however, the d.m.e. is used as an indifferent electrode in a vacuum tube galvanometer circuit.

In Fig. 16 the E_h is shown by the dropping mercury electrode vacuum tube galvanometer record and the absorption of $\lambda = 5490$ A is shown by the line (irregular due to electrical disturbances) of the spectrophotographic galvanometer. This is a typical record for 20 p.c. yeast in phosphate buffer (pH 6.8), washed, and then oscillated for twenty-four hours in thin layers in contact with air in Erlenmeyer flasks, i.e., until a low endogenous metabolism was reached. This endogenous yeast suspension was saturated with air, poured into the cuvette of the spectrophotometric apparatus, and a d.m.e. in a vacuum tube galvanometer circuit had its electrode directly in the cuvette. The yeast was allowed to consume the O_2 in solution and to develop a low E_h with-
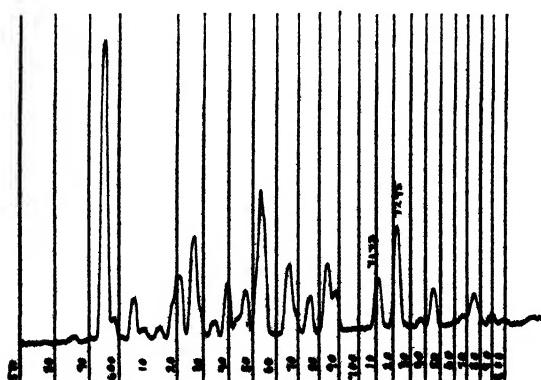
FIGURE 13.
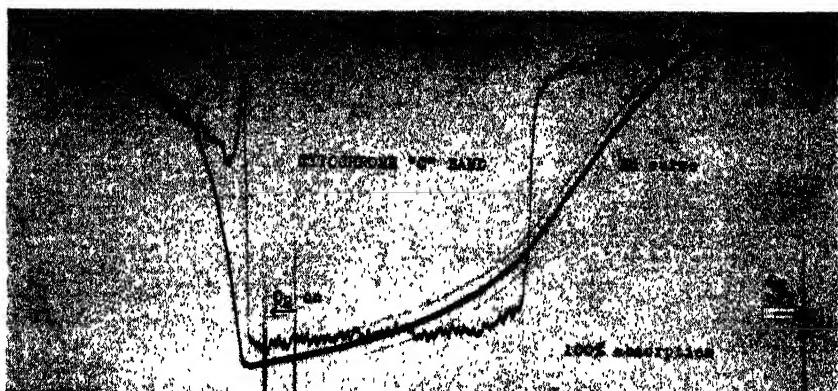
FIGURE 14.
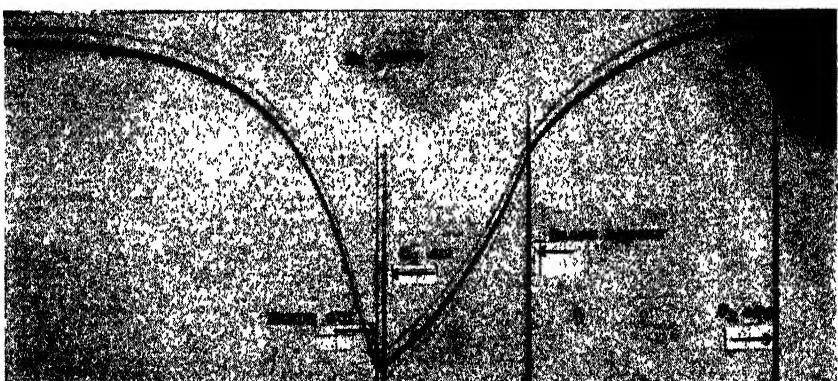
FIGURE 15.
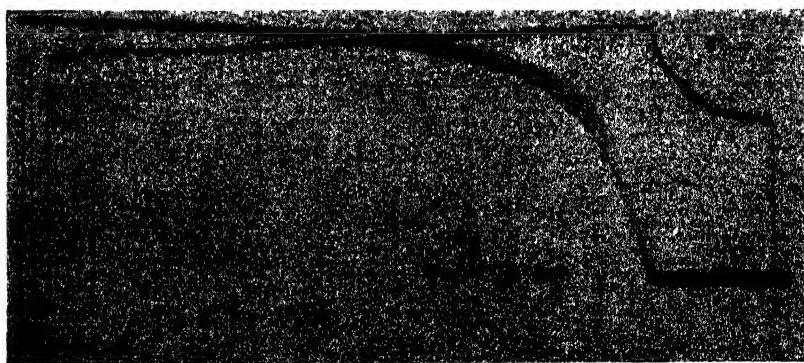
FIGURE 16.

out any agitation of the solution. The figure shows that no reduction of cytochrome occurs, in endogenous metabolism, until the E_h falls to a minimum value, whereupon cytochrome is reduced gradually.

Yeast (15 p.c.) with a high exogenous metabolism in buffer (pH 6.8) + substrate, and with gases bubbled through an alundum bubbler supplying oxygen continuously to the cells in the cuvette while simultaneously the light transmission and E_h are determined as in the case above, gives a record like Fig. 17. In this case, however, λ changes to values indicated by the abscicca as the result of the continuous turning of the prism of the monochrometer. Three runs through the spectral transmission measurement from 6,000 A to 5,000 A are shown in the figure. For each run, which required about 500 seconds, the gas mixture bubbling through was set at such a value of O_2 tension that cytochrome-c would be reduced to the degree desired, and during the run nothing was changed except λ. The record of each run is therefore entirely automatic. The successive runs followed on each other, with only a few minutes gas equilibration, to the new degree of reduction of cytochrome-c to be established — otherwise nothing was changed. The gases used were, respectively, for the three records (reading from top to bottom at λ 5490 A): air, air and tank nitrogen mixture, tank nitrogen of high purity. In each run the degree of reduction of the three cytochromes is shown by the steady lines of the spectrophotometric galvanometer. It is obvious that the relative degree of reduction of all of the three cytochromes is the same in any one run. Fig. 18 also illustrates this point, showing two sets of curves. Here the conditions were essentially the same as in Fig. 17 except that there is no determination of E_h and the successive gas mixtures used were air, air and tank nitrogen, nitrogen, and finally no gas at all. Again the relative
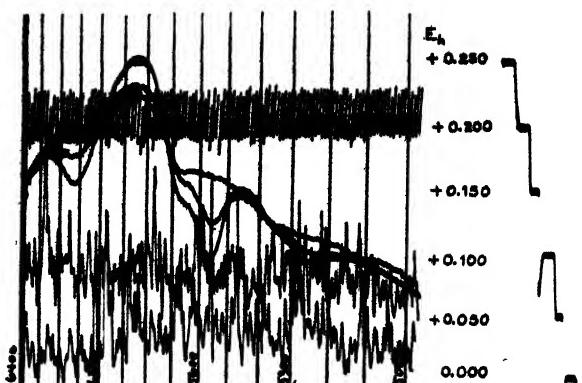
FIGURE 17.

degree of reduction of the three cytochromes is the same in each run.

To return to Fig. 17, the E_h accompanying each run is shown by the d.m.e. vacuum tube galvanometer record. The E_h records are in the same order as the spectrophotometer records at $\lambda =$ 5490 A. The E_h is seen to be more steady at 0.23 than at the lower values. Low E_h values are characteristically more sensitive to fluctuations. This is probably due to the sensitively balanced dynamic equilibrium which exists between the dehydrogenation processes and the oxidation of cytochrome in this range of E_h, which is upset by local changes in O_2 tension accompanying the bubbling. In spite of these fluctuations, the average E_h may be readily estimated. The E_h corresponding to approximately half reduction of the three cytochromes lies between 0.10 and 0.15, and complete reduction between 0.10 and 0.05. The E'_o indicated for cytochrome-c by this work, like that in Table III, is close to Green's value of 0.123. The values indicated for cytochromes-a and -b are the same as for -c, as was also the case in the spectrophotometric work shown in Fig. 12, discussed above.
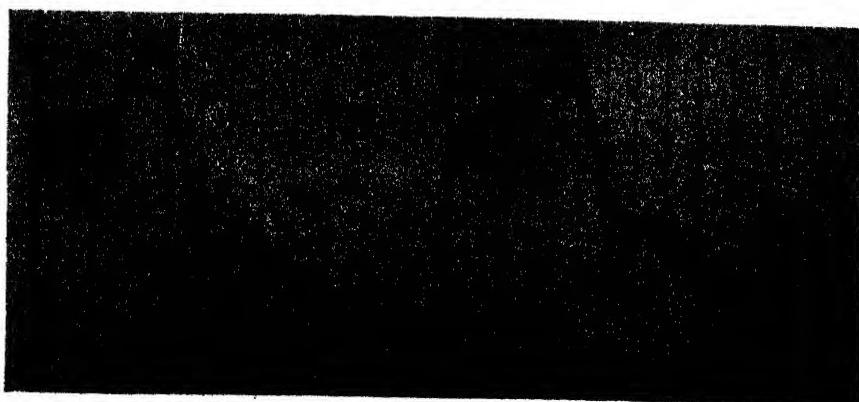
FIGURE 18.

Tamiya and Ogura (53), by observing the order of appearance of cytochrome bands with a hand spectroscope, believed that

$$\frac{\text{Red -b}}{\text{Ox -b}} > \frac{\text{Red -a}}{\text{Ox -a}} > \frac{\text{Red -c}}{\text{Ox -c}}$$
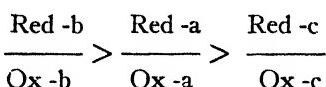
The authors found that the E'_0 of the respective cytochromes were in the order -c, -a, -b, with -c the most positive. However, it should be pointed out that relative intensity of absorption and relative spectral sensitivity of the eye would make such an estimate of little quantitative value, if not erroneous.

The "coupled E'_0," (37) of cytochrome-c from heart muscle in pure solution, according to Stotz, Sidwell, and Hogness (49), is 0.262; and the E'_0, according to Wurmser and Filitti-Wurmser (47), is 0.253. Ball (48), by coupling systems, found 0.29 for -a and —0.04 for -b in heart muscle *brei*. If we assume these values to be correct and to apply to cytochromes -a, -b, and -c *in living yeast cells*, then we would expect a different degree of reduction of the different cytochromes calculated in the usual way, as by Tamiya and Sato (54). We might explain the equal reduction of all three cytochromes (Baumberger, 55) as being due to their occurrence in a molecular aggregate of all three, which is reduced or oxidized as a whole, and that various ratios of these fully oxidized and fully reduced complex aggregates would occur. This would necessitate reduction by several mediator molecules at the same time, and therefore seems quite unlikely, although the dehydrogenase - carrier - cytochrome - oxidase complex described by Stern (56) could furnish such a mechanism provided one assumed an electronic transfer through hydrogen bonds as pictured by Borsook (9). Another possibility is that the rates of oxidation and reduction of the three cytochromes are quite similar and therefore

the statistical expectancy would be the same. In view of the functioning of oxidase in the case of cytochromes-a and -c and not in the case of -b (Keilin, 40), this would seem unlikely, although according to the activation theory of rates of reaction discussed by Barron (25) the reaction rate is proportional to the free energy of the reaction. The rate of oxidation of cytochrome-b would be greater than -a or -c in the absence of the oxidase. A third possibility might be that in the molecular aggregate, all three cytochromes have the same E'_0. It does not seem possible to choose between these three possibilities. The facts brought out which have the closest bearing on the general arguments of this paper are that the degree of reduction of cytochrome varies with the E_h when rapid exogenous metabolism is going on.

VII. THE RELATION OF OXYGEN TENSION TO THE REDUCTION OF CYTOCHROME

Applying the dropping mercury electrode method of determining oxygen tensions by introducing the cathode into the spectrophotometric cuvette and connecting it to a calomel half cell, records of simultaneous changes in pO_2 and light transmission were obtained. With an endogenous yeast preparation, similar to the one used in Fig. 16, Fig. 19 was obtained. Here by a calibrated shunt the sensitivity of the galvanometer could be varied to a known degree during the course of consumption of all the oxygen in solution so that in the final phase 1 mm. is equivalent to 3×10^{-4} atmospheres of oxygen. The spectrophotometer (set at $\lambda = 5490$ A) gave a record showing increased light absorption to occur only when, and at the same instant as, the oxygen had been completely removed.

Exogenous 8 p.c. yeast in buffer (pH 6.8), however, showed a partial reduction of cytochrome-c when some oxygen still remained. In Fig. 20 pO_2, E_h and absorption at $\lambda = 5490$ A are recorded simultaneously. In the left curve

FIGURE 19.
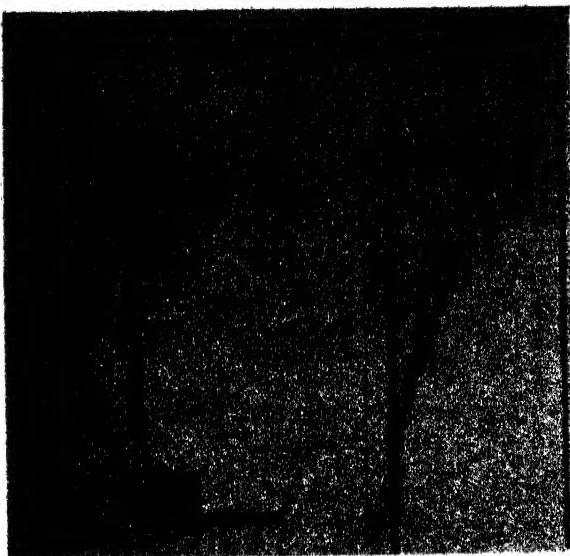
FIGURE 20.

the oxygen was removed partially by consumption and at the same time by sweeping through with nitrogen containing a few tenths of a percent of oxygen. E_h reached its lowest value simultaneously with the disappearance of oxygen, but the reduction of cytochrome started before all the oxygen had been used up, in fact while 1.2 p.c. oxygen remained. This is shown by vertical lines drawn from the point where reduction of cytochrome first appears.

The right hand portion of the record shows the reoxidation of cytochrome by bubbling air. Here complete reoxidation is only reached after about 1.2 p.c. O_2 is present.

The rate of oxygen consumption of the exogenous cells may be as much as 100 times as great as that of endogenous cells. In maintaining such a high metabolic rate an increased degree of reduction of cytochrome, and probably also of various mediators, occurs. This is indicated by
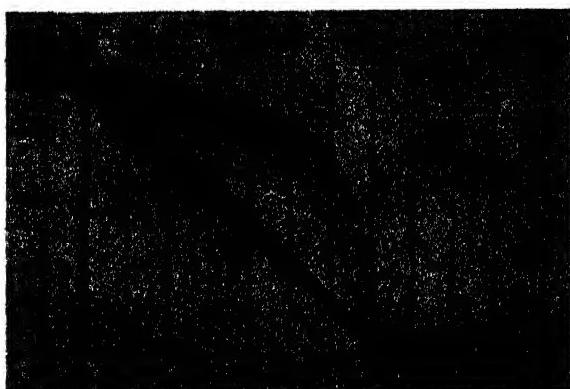
FIGURE 21.

many of the facts previously brought out. One of these factors may be the slowly oxidizable material discussed below.

The occurrence of some compound which is only slowly oxidized by air and which modifies the relation of E_h to oxygen pressure is suggested by the following experiments. Fig. 21 shows the perfect coincidence of zero oxygen with lowest E_h. The E_h calibrations are shown by dashes at the left in 50 millivolt units from E_h 0.300 down to 0.100. Abscissas represent equal units of time (approximately 40 seconds). $\Delta E_h/\Delta pO_2$ becomes progressively larger in successive time intervals. O_2 changes from 21 p.c. to 0 p.c. in a perfectly linear relation with time. The perfect coincidence in time of E_h minimum and O_2 disappearance is also shown in Fig. 19. If, however, a yeast cell suspension is not merely equilibrated with air but is shaken for a period of several minutes at slightly more than an atmosphere of air, such coincidence of E_h and pO_2 minima does not occur, but in its place E_h reaches the minimum some time after the oxygen has all disappeared. In Fig. 22, 0.6 p.c. yeast + glucose was used, and the E_h and O_2 determinations were made simultaneously.

FIGURE 22.

Time proceeds from left to right. The galvanometer sensitivity for O_2 determination is initially 3/100 and then is changed to 2/10 in each case. In the first graph E_h does not reach a minimum at the same time as O_2 as a result of more prolonged oxygenation of the suspension, while in the second curve simultaneity is approached in the identical yeast suspension by equilibrating with air for the minimum time necessary. Vertical lines drawn on the photograph from the point where zero O_2 is reached show the corresponding E_h attained.

There is a similar coincidence of E_h minimum with cytochrome-c reduction in Fig. 16 and lack of coincidence in Fig. 23; these are procured by similar oxidative treatments as above.

The possible role of this "slowly oxidizable material" will be considered later.

FIGURE 23.

VIII. THE RELATION OF Q_{O_2} TO THE TENSION OF OXYGEN.

A. When Oxygen is in Physical Solution.

Fig. 5, 6, 19-22 show photographically that Q_{O_2} is not affected by pO_2 from 0.21 down to 0.0003 atmospheres and possibly even lower. If there exists a critical oxygen tension it must certainly be below 0.0003 atmospheres.

While the Q_{O_2} remains unaffected by pO_2, in the range studied, changes in the mediator systems occur, as described in previous sections, which may facilitate the transfer of hydrogen and/or electrons to oxygen in such a way as to keep Q_{O_2} constant.

B. When Bound Oxygen is Present.

In yeast suspensions the oxygen consumption rate is independent of the oxygen pressure; however, that is not the case if there is present bound oxygen, in the form of oxyhemoglobin. When bound oxygen is present there is an *increase* in the rate of oxygen consumption as the oxygen pressure *falls*. This can be calculated from measurements of records similar to Fig. 24. The

determination of oxygen tension and light transmission are shown. Freshly drawn sterile defibrinated monkey blood was added to Ringer's solution, and yeast cells were added with glucose so that in a suitable time the oxygen would be consumed. A fixed wavelength of light was used which gave a maximum absorption by reduced hemoglobin and minimum absorption by oxyhemoglobin, and which at the same time showed no difference in absorption by reduced and oxidized yeast suspensions. In Fig. 24 the change in oxygen pressure with time (progressing to right) is accompanied by a change in oxyhemoglobin; and finally the oxyhemoglobin is fully reduced when the oxygen is completely used up by the yeast cells. The spectrophotometric data plotted against oxygen tension give the typical oxygen dissociation curve of oxyhemoglobin (16) shown to the left in Fig. 25. This curve is the average of five records on three bloods.

If one assumes that the oxygen is being consumed at a uniform rate regardless of the pressure of oxygen, it would seem logical that the oxygen dissociation curve could be calculated from the time factor. The procedure followed is to meas-
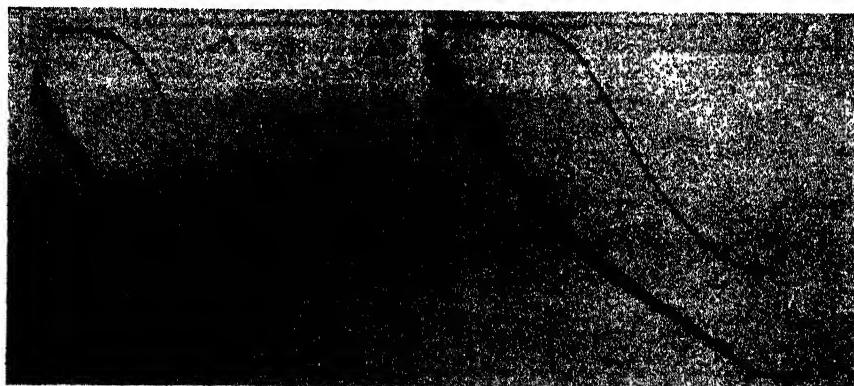
FIGURE 24.
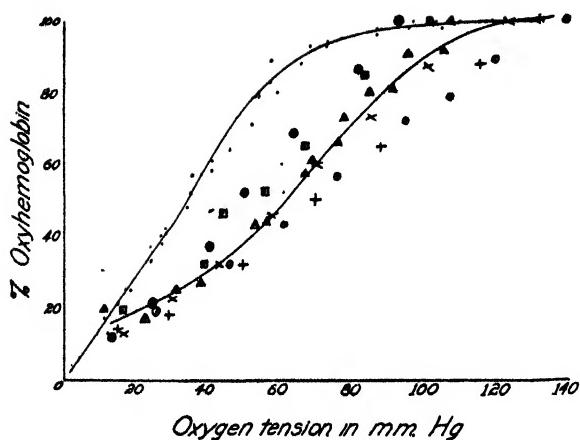
FIGURE 25.

ure from the photographic record the time necessary to consume the oxygen still present at a series of oxygen tensions, and from these to estimate how much oxygen is left. Subtracting from the oxygen present the amount in physical solution, the remainder will be the bound oxygen at the observed tension. The dissociation curve thus obtained is shown to the right in Fig. 25. Here the points are from seven different bloods. The curve is displaced decidedly to the right and does not represent the true oxygen dissociation curve of oxyhemoglobin. To make the "time" method agree with the correct spectrophotometric method it is necessary to postulate that the rate of oxygen consumption in yeast increases as the pressure of oxygen falls so that, for example, at the center of the curve Q_{O_2} about doubles. Q_{O_2} calculated on the assumption that it is constant, i.e., the average Q_{O_2}, must be multiplied by 1, 1.2, 1.7, and 1.9 respectively at pO_2 120, 90, 60, and 30 mm. of mercury in order to obtain by the "time method" an oxygen dissociation curve for oxyhemoglobin which is correct.

The observation that bound oxygen is used up more rapidly than would be expected agrees with the observations of Neumann (57), Emödi and Sarkamy (58), and Emödi (59), that the Q_{O_2} of yeast cells determined by hemoglobin reduction is greater than the Q_{O_2} determined by the Barcroft-Warburg technique. The following experiment is typical of many made in this laboratory. The iron content of fresh sterile defibrinated monkey blood was determined colorimetrically (60, page 670) to be equivalent to 18.36 volume per cent O_2. The time required for a 0.03 p.c. yeast, 1 p.c. glucose suspension to completely consume all the oxygen was determined for a 10-fold dilution of blood in which the total oxygen would be about 4 times the soluble oxygen, and for a 250-fold dilution in which the total oxygen

would be only slightly above the soluble oxygen. The former was found to require 600 seconds, and the latter 325 seconds for oxygen consumption. In other words, when there is much bound oxygen present the Q_{O_2} is accelerated to about twice normal. This acceleration of Q_{O_2} by bound oxygen is not due to any catalytic effect of hemoglobin or oxyhemoglobin because no such action of erythrocytes or hemolyzed blood is demonstrable in Warburg experiments.

IX. Conclusions.

The purpose of this paper is to show the relation of E_h to Q_{O_2}. The following pertinent facts have been brought out:—

1. E_h decreases with decrease in pO_2.
2. E_h decreases more and more rapidly as pO_2 decreases.
3. When E_h is greater than 0.25, Q_{O_2} decreases.
4. Q_{O_2} is independent of the pO_2 of solutions.
5. Cytochrome may be reduced in the presence of oxygen when the Q_{O_2} is high.
6. A slowly oxidizable material is formed during the reduction of yeast.
7. Q_{O_2} increases with decrease in pO_2 when bound oxygen is present.

To use these facts in an explanation of the relation of E_h to Q_{O_2}, the terminology and considerations of Warburg and Kubowitz (1) are useful. These may be stated as follows:—

A = respiration (*Atmung*) at reduced pressure O_2.

A_o = respiration (*Atmung*) in air.
In a substrate saturated cell,

$$-\frac{d\mathrm{Fe}^{++}}{dt} = 0 = B \cdot pO_2 \cdot \mathrm{Fe}^{++} - Z' \cdot \mathrm{Fe}^{+++}$$

B = constant for oxidation rate of Fe^{++}/min. $\times$ atm.

Z' = reduction of Fe^{+++} by cell.

Then since Z'/B does not vary with pO_2 (i.e., above 1×10^{-5} atm.) for micrococcus,

$A = Z' \cdot$ total iron.

Leaving Warburg now, the assumption may be made that the drop in E_h represents an increase in Fe^{++} (using the terms Fe^{++} and Fe^{+++} in a loose sense to mean any catalyst or mediator). The whole subject may be approached differently to say that even at magnitudes of 0.1 atm. of oxygen

$$Fe^{+++}/Fe^{++} \propto pO_2$$

so that

$$Fe^{++} \cdot pO_2 \cdot B = Fe^{+++} \cdot Z'$$

since when

$$p_1O_2 < p_2O_2 \text{ then } Fe^{++}_1 > Fe^{++}_2$$
$$\text{and } Fe^{+++}_1 < Fe^{+++}_2.$$
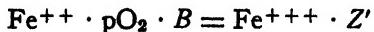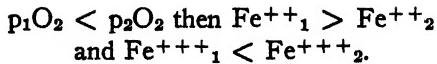
Then to keep A constant, Z' must increase. How can Z' increase?

Assume that the degree of reduction of the various systems of the cell is determined by the E_h. Then, although the removal of activated hydrogen and/or electrons is continuing at the same rate, at low pO_2, the reductants on hand (cytochrome, yellow enzyme, etc.) are increased in concentration so that the transfer of electrons to Fe^{+++} will remain at the normal number per hour in spite of a decrease in concentration of Fe^{+++}. This could occur by a more effective hydrogen transfer system resulting from the "overlapping phenomenon" as follows:—

Let $E'_{o_1} = E'_{o_2} + 0.060.$

Then, if $n = 1$ in each case and the concentrations of the two systems are equal, when

$$\frac{Ox_1}{Red_1} = \frac{9}{1} \text{ then } \frac{Ox_2}{Red_2} = \frac{9.9}{0.1} \text{ and } Ox_1 \cdot Red_2 = 0.9$$

and when

$$\frac{Ox_1}{Red_1} = \frac{5}{5} \text{ then } \frac{Ox_2}{Red_2} = \frac{9}{1} \text{ and } Ox_1 \cdot Red_2 = 5$$

Such an overlapping system of hypothetical mediators could transfer electrons about 3 times as fast if the final oxidant remained constant in concentration or could keep up a constant rate of transfer even if some commensurate decrease in concentration of the final oxidant occurred.

It would be tempting to assign this function to the three cytochromes, but the evidence here presented that they are all proportionately reduced does not seem to support it.

The increase in Q_{o_2} with decrease in pO_2 when bound oxygen is present gives some support to these theories. Here, with a decrease in pO_2 and E_h, increased reduction of mediators probably occurs and this facilitates electron transfer. The Q_{o_2} can then increase because of the oxygen available in oxyhemoglobin.

The evidence presented suggests that the degree of reduction of mediators varies with oxygen pressure in such a way as to keep the rate of oxygen consumption constant in yeast cell suspensions.

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Discussion

Dr. Velick: The experiments in which the relative amounts of oxidized and reduced cytochrome were measured at different oxygen tensions indicate, as you point out, that the mechanism which maintains the rate of oxygen consumption at lowered oxygen tensions may be related to the product of the concentrations of reduced cytochrome-c and oxidized cytochrome oxidase. Unfortunately, in the experiments in which the bands of reduced cytochrome appeared at relatively high oxygen tensions, the rate of oxygen consumption was not directly followed, but instead the oxidation-reduction potential of the external medium was determined. This quantity, although affected by the oxygen tension, is related to it in a complicated, non-linear way, so

that from it we can get only a rough idea of the amount of oxygen present. In the experiment in which the oxygen consumption was measured with the dropping mercury electrode the bands of reduced cytochrome did not appear until oxygen tension was almost zero, so that in this case the proposed kinetic mechanism breaks down. The complicating factor, of course, is that in this experiment the suspension was not stirred.

Dr. Baumberger: An appreciable percentage of reduced cytochrome occurs, in the presence of measurable oxygen, only when strong exogenous metabolism exists. This is shown in Fig. 20. When only endogenous metabolism exists, all the substrate activated can be oxidized without any appreciable reduction of cytochrome, mediators, etc.

Dr. Velick: These differences between stirred and unstirred suspensions suggest inhomogeneities in the suspensions, and also in the cell population. The latter difficulty might arise if there were a significant proportion of budding cells in the preparation, since the budding cells might be expected to be metabolically different. Certain bacteria after fission are, for a time, markedly different in their metabolic behavior from mature organisms. This is also true in the case of young and mature blood cells. I would, therefore, like to know the nature of the yeast preparation, the duration of the experiment, and whether there are any data on the changes in metabolism of yeast cells during their cycle of development.

Dr. Baumberger: The duration of most of the experiments on E_h, pO_2 and Q_{O_2}, etc. was only about 1 to 15 minutes. Cell counts, viability tests, and budding counts were made in the early part of the work (1933-34) but no appreciable changes occurred. After all, the cells are suspended in a nitrogen-free medium with only glucose as a substrate; under these conditions yeast does not grow.

Dr. Hogness: As I recall, the potential of the oxygen electrode at pH 7 is about 0.80, and that of the cytochrome is about 0.27, so the difference is about 0.53. Now, if one calculates the pressure of the oxygen which is in equilibrium with the cytochrome, I believe one finds the cytochrome should not be appreciably reduced until the oxygen pressure is reduced to an extremely small value. Your observation that there is no reduced cytochrome present until the oxygen is reduced to a very small figure seems to be compatible with such a calculation.

Dr. Baumberger: I think your mathematics are correct, but I doubt if they apply to this case where there is a dynamic equilibrium involving not only cytochrome and oxygen, but also mediators which continue to reduce cytochrome. No thermodynamic equilibrium could exist between cytochrome and irreversibly reduced oxygen.

Mr. MacLeod: Suppose you were using a tissue which had a high aerobic fermentation or an aerobic glycolysis; what would you expect the alcohol or lactic acid to contribute to the result?

Dr. Baumberger: Such metabolites would not affect the Q_{O_2} determination by the dropping mercury electrode, as they may not be reducible at the electrode at the single potential used in the O_2 determination. If reduction of some compound (other than oxygen) did occur at the electrode, a correction could be made for the resulting current as it would be included in the zero oxygen current.

We have also made Q_{O_2} determinations on *B. coli*, finely minced heart muscle, etc. with good results. The cells must remain well suspended in this method unless the air is circulated from electrode vessel through the tissue containing vessel in a closed circuit. This is a suitable procedure for slices, small animals or eggs.

Müller has been working with this method with animal tissues. Perhaps he can give us some additional information.

Dr. Müller: My experiments have been on adrenal tissue and are preliminary; the only thing I can say is that the cortex respires much faster than the medulla, which one would expect. I have not done any quantitative work along these lines.

About the oxygen curve—it is an experimental fact that we reduce oxygen at the dropping mercury electrode at a potential far more negative than that given to the oxygen electrode. I do not know how it can be explained, but it can be shown that the reduction goes in two steps, first to hydrogen peroxide and then to hydroxyl ion. The difference between the two potentials is in the neighborhood of 0.7 v., but the reason for this value I do not know. We can increase the second step by adding hydrogen peroxide and we can remove it entirely by adding a catalase. The oxidation-reduction potential of this system cannot be compared with that of a reversible system because we know the process is not reversible in a thermodynamic sense. We know also that it does not have the dependence on pH that one would expect from the formulation. Nevertheless, the reduction of oxygen at the dropping mercury electrode is a fact, and we cannot overlook it.

THE INHIBITION OF GLYCOLYSIS

CHALMERS L. GEMMILL

Glycolysis may be defined as the enzymatic conversion of carbohydrate into lactate. In this review, however, the term will be restricted to the conversion of glycogen to lactate in muscle extracts and reference will be made to glycolysis by other tissues and fermentation by yeast only when the necessary data is lacking for muscle extracts. The enzymatic conversion of glycogen to lactate by muscle extracts is a complicated process involving many enzymes and coenzymes. A tabulation of the recent work is given in Table I. It is obvious that the opportunities for blocking this process are many, for inhibition may be produced by the agent combining with the substrate or with one or more of the enzymes, coenzymes, or intermediate products. In this review only specific inhibitors will be discussed; their site of action will be determined and their application to skeletal muscle will be given in relation to the current scheme of glycolysis.

Amylase. If a competing enzyme or enzyme system is present, lactate formation by the method outlined above may be inhibited. Winfield and Hopkins (7) discovered an example of this competition, for they demonstrated that pancreatic extracts suppressed the formation of lactate. Case and McCullagh (1) compared the activities of these extracts with those of amylase and found a close correspondence. Ronzoni (5) showed that this inhibitor not only stopped lactate production but also esterification of phosphate. Since there was no loss of total carbohydrate, the assumption was made that the inhibition involved the first step in the glycolytic system, namely, the phosphorylation of glycogen. McCullagh (4) gave additional evidence that this was the point of inhibition, for he demonstrated that there was no disappearance of free phosphate in the presence of fluoride. Harrison and Mellanby (3) observed that lactate formation from hexosediphosphate was inhibited by pancreatic extracts but not from hexosemonophosphate. These facts are difficult to explain on the basis of the present scheme of glycolysis. Gill and Lehmann (2) recently demonstrated that the inhibitory effect of amylase was abolished when sufficient glycogen was added to saturate both systems, thus furnishing final proof that the effect was produced by competition between the two systems for the substrate. Since muscle extracts contain considerable amylase (Willstätter and Rohdewald, 6) the competition for the carbohydrate substrate by this enzyme and by other enzyme systems must be considered in evaluating the effect of any agent on the disappearance of glycogen and the formation of lactate.

Fluoride. One of the most valuable inhibitory

agents on glycolysis has been the fluoride ion, for it has been through its activity that many of the details of our present glycolytic system have been revealed. The first quantitative study of its action in muscle extracts was made by Embden and Haymann (10). They observed that fluoride, in addition to inhibiting lactate formation, caused a disappearance of free phosphate in the incubated mixture. The latter reaction was accelerated by the addition of glycogen but not by the presence of dextrose or maltose. Hexosediphosphate was formed in the extracts containing fluoride. These authors also observed that the rate of esterification was dependent on the concentration of fluoride and that dilutions as low as M/1280 had slight inhibitory activity. These observations were confirmed and extended by many observers. Davenport and Cotonio (8) correlated the disappearance of phosphate with the carbohydrate changes and found that the disappearance of free phosphate was associated with an actual decrease in total carbohydrate. Lohmann (18) demonstrated that a phosphate ester was produced in the fluoride extracts, which was resistant to acid hydrolysis and the amount of this ester formed was dependent on the amount of glycogen, starch, or the Harden-Young ester added to the muscle extracts.

These observations were explained by the isolation of phosphoglycerate from similar extracts by Embden and Deuticke (11). The formation of this ester, difficult to hydrolyze in acid, accounted for the disappearance of free phosphate and the decrease in total carbohydrate. These authors showed that an extract with fluoride allowed hexosediphosphate to go to phosphoglycerate but this compound, in turn, could not be converted into pyruvate and phosphate. If, however, pyruvate was added to such mixtures, lactate formation occurred. It was this observation that helped Embden and his associates (11) to connect hexosediphosphate, the triosephosphates and phosphoglycerate with a coordinated scheme of glycolysis.

Lohmann and Meyerhof (19) divided the transformation of phosphoglycerate to pyruvate and phosphate into two separate reactions and showed that the first reaction was more sensitive to fluoride inhibition than the second.

$$\text{Phosphoglycerate} \underset{}{\overset{\text{Enolase}}{\rightleftharpoons}} \text{Phosphopyruvate}$$

$$\text{Phosphopyruvate} \xrightarrow{\text{Phosphatase}} \text{Pyruvate} + \text{Phosphate}$$
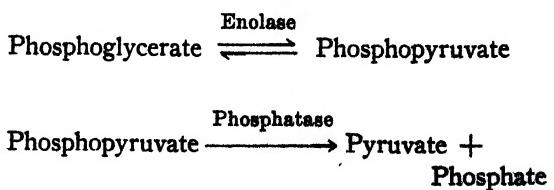
TABLE I

Current scheme of glycolysis.

(Inhibitors placed in parenthesis.)

Reaction	Enzyme	Coenzyme
1. Glycogen + phosphate $\rightleftharpoons$ glucose-1-phosphate (Amylase, glucose, phlorizin)	Phosphorylase	Adenosinemonophosphate
2. Glucose-1-phosphate $\rightarrow$ glucose-6-phosphate	Phosphoglucomutase	Mg^{++}, Mn^{++}, Co^{++}
3. Glucose-6-phosphate $\rightleftharpoons$ fructose-6-phosphate	Phosphohexoisomerase	
4. 2 Hexose-6-phosphate + adenosinetriphosphate $\rightleftharpoons$ adenosinemonophosphate + 2 hexose-1-6-diphosphate	Phosphorlyase	Mg^{++}
5. Hexose-1-6-diphosphate $\rightleftharpoons$ 2 triosephosphate	Aldolase	
6. Dihydroxyacetonephosphate $\rightleftharpoons$ 3-glyceraldehydephosphate	Phosphotrioseisomerase	
7. Triosephosphate $\rightleftharpoons$ 3-phosphoglycerate (Monoiodoacetate, oxidizing agents)	Triosephosphate dehydrogenase	Coenzyme [Co $\rightarrow$ CoH$_2$] (snake venom) adenosinemonophosphate, creatine
8. 3-Phosphoglycerate $\rightleftharpoons$ 2-phosphoglycerate	Phosphoglyceratemutase	
9. 2-Phosphoglycerate $\rightleftharpoons$ phosphopyruvate (Fluoride, citrate)	Enolase	
10. Phosphopyruvate $\rightarrow$ pyruvate + phosphate (Oxalate, citrate)	Phosphatase	Adenosinemonophosphate
11. Pyruvate $\rightleftharpoons$ lactate (Sulfite)	Lactate dehydrogenase	Coenzyme [CoH$_2$ $\rightarrow$ Co]
12. Adenosinemonophosphate + 2 creatinephosphate $\rightleftharpoons$ adenosinetriphosphate + 2 creatine	Phosphorylase	Mg^{++}
13. 2 Phosphopyruvate + adenosinemonophosphate $\rightarrow$ adenosinetriphosphate + 2 pyruvate	Phosphorylase	Mg^{++}

Therefore, the actual point of inhibition of fluoride is on the enzyme catalysing the transformation of phosphoglycerate to phosphopyruvate.

Since the enolase does not require a coenzyme or an external inorganic component, the action of fluoride must be on some part of the protein molecule of the enzyme itself. Lipmann (15) offered an attractive theory to explain the activity of fluoride. His idea was that fluoride reacts with a trivalent heavy metal component in the enzyme to form an easily dissociatable complex, similar to fluormethemoglobin. His evidence was indirect but striking. Other substances such as HCN and H_2S (Warburg, 22) inhibit yeast fermentation and react with methemoglobin. Nonbiological systems catalyzed by heavy metals were inhibited by fluoride. The fluoride inhibition was reversible and a dissociation curve of fluormethemoglobin was constructed from experimental data. Additional evidence that iron may be involved was supplied by Rothschild (20) who compared the inhibition of fluoride on glycolytic systems to that on lipase activity. He found, as Lovenhart and Peirce (17) did many years ago, that the inhibitory action of fluoride on lipase was reversible and in addition observed that other substances known to form iron complexes inhibited lipases. It may be that the enolase has a ferric or heavy metal component.

There have not been many applications of fluoride inhibition to muscular activity. Lipmann (16) observed that muscles taken from frogs injected with fluoride went into contracture after a few stimulations. Under these conditions, lactate production was diminished and phosphocreatine breakdown increased. Several observers (Dickens and Simer, 9; Sellei and Jany, 21; Ewig, 12) have demonstrated that respiratory activity of cells was not inhibited to the same degree as the glycolytic processes. It is not surprising that glycolysis is more sensitive to fluoride action than respiration. If the only site of action is on the transformation of phosphoglycerate to phosphopyruvate, the triosephosphate may still be oxidized by the coenzyme + cytochrome + O_2 system. Without the cytochrome system or under anerobic conditions the coenzyme should be reduced and not reoxidized in fluoride poisoned extracts. However, Lennerstrand (13) demonstrated that in the system apozymase + cozymase + glucose + hexosediphosphate + pyocyanin + phosphate the cozymase was inactivated when fluoride was added. He claimed that the inactivation was due to the fluoride inhibiting the phosphorylating activity of cozymase. These results suggest that a second site of inhibition may be present. It is surprising, in view of the knowledge of the inhibitory action of fluoride, that more use has not been made of this special property in the study of physiological processes in intact cells.

Monoiodoacetate. Soon after the discovery of Lundsgaard (37) that monoiodoacetate prevented lactate formation in skeletal muscle during stimulation, Lohmann (36) investigated its action on muscle extracts and found that in addition to lactate inhibition there was no disappearance of added Harden-Young ester and that there was partial inhibition of esterification of glycogen. The inhibition was not reversible and was only complete after a considerable period of time. These facts pointed to a different site of inhibition than that of fluoride, especially as Embden and Deuticke (28) showed that the oxido-reduction of hexosediphosphate to phosphoglycerate and glycerophosphate was sensitive to the action of monobromacetate. When this inhibitor was added to muscle extracts only a very small amount of hexosediphosphate disappeared and no trace of phosphoglycerate was found. Thus these observations pointed to the inhibition occurring before the formation of phosphoglycerate. Dixon (27), in a comparative study of the sensitivity of dehydrogenases to monoiodoacetate, observed that alcohol and triosephosphate dehydrogenases from yeast were extremely sensitive to this inhibitor. Green, Needham and Dewan (31) extended these observations to muscle extracts and found that the reactions of triosephosphate, triose or α-glycerophosphate with pyruvate were inhibited by small concentrations of monoiodoacetate. At the time of publication of this paper, these authors thought that they were dealing with mutases which had different properties from dehydrogenases. However, if the cozymase factor was added to these systems (Adler and Hughes, 23; Green, Needham and Dewan, 30) the enzymes involved in the dismutation had properties comparable to typical dehydrogenases. The oxygen uptake of the system mutase + coenzyme + coenzyme factor + methylene blue + hexosediphosphate was inhibited by M/200 iodoacetate. If the cytochrome system (Needham and Lu, 43) was used in place of methylene blue an inhibition of oxygen uptake likewise occurred. Meyerhof and Kiessling (42) also reported that the reaction of pyruvate with glycerophosphate was inhibited by small amounts of monoiodoacetate.

Monoiodoacetate inhibition has recently been reinvestigated by Adler, von Euler and Günther (24). By following the reduction of the coenzyme spectrometrically, they were able to determine that the triosephosphate dehydrogenase from yeast was more sensitive to inhibition than the alcohol dehydrogenase. These authors found also that the reaction of α-glycerophosphate + pyruvate was not affected by monoiodoacetate. They showed that coenzyme and the coenzyme factor

were not involved. The action of the inhibitor was on the triosephosphate dehydrogenase and not on the associated phosphorylation for in the presence of arsenate, which separates the phosphorylation from the oxido-reduction, monoiodoacetate inhibition also occurred.

Since the triosephosphate dehydrogenase is associated through the coenzyme with the lactate dehydrogenase by the following reactions,

Triosephosphate + coenzyme $\underset{\substack{\text{triosephosphate} \\ \text{dehydrogenase}}}{\rightleftharpoons}$

phosphoglycerate + reduced coenzyme.

Pyruvate + reduced coenzyme $\underset{\substack{\text{lactate} \\ \text{dehydrogenase}}}{\rightleftharpoons}$

lactate + coenzyme.
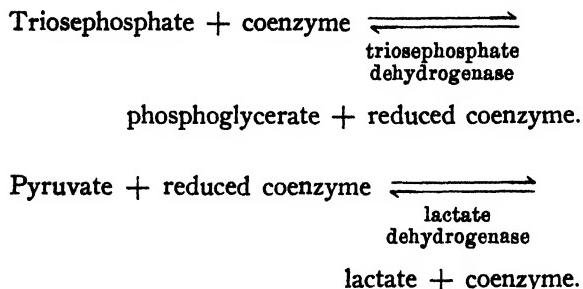
it is of interest to compare the activity of monoiodoacetate on lactate dehydrogenase with its effect on triosephosphate dehydrogenase. Green and Brosteaux (30) found that M/50 iodoacetate had no effect on lactate dehydrogenase while Dixon (27) observed 62 p.c. inhibition with M/100 iodoacetate. Adler, von Euler and Günther (24) reported that this enzyme was very insensitive to iodoacetate. Green, Needham and Dewan (31) also showed that the system mutase + coenzyme + coenzyme factor + methylene blue + lactate + cyanide was not inhibited to M/200 iodoacetate. These results illustrate the difficulty of comparing inhibitory action on the basis of concentration of the inhibitor. The activity of monoiodoacetate depends not only on the amount and on the presence of other substances besides the enzyme in the mixture but also on the time of incubation. In spite of these difficulties of comparison, the existing evidence points to a much greater sensitivity of the triosephosphate dehydrogenase than of the lactate dehydrogenase to monoiodoacetate.

There has been considerable speculation concerning the effect of monoiodoacetate on inhibition of the triosephosphate dehydrogenase. Dixon (27) and von Euler (24) demonstrated that the action was not on the coenzyme but on the protein component of the enzyme system. The most attractive theory is that monoiodoacetate reacts with a sulfhydryl group in the triosephosphate dehydrogenase. This idea will be discussed in another section.

This inhibitor has been extensively used in the study of glycolysis of intact tissue. In a muscle poisoned with monoiodoacetate there is no lactate production associated with activity. There is, however, an accumulation of hexophosphate and

during recovery (Cori, Cori, and Hegnauer, 26) hexosemonophosphate is reconverted into glycogen. Triosephosphate does not accumulate during contraction of poisoned muscles (Sacks, 44). The resting oxygen consumption of poisoned muscles is normal but decreases after several hours (Saslow, 45). The ratio of oxygen consumption during work to tension developed is about the same as in a normal muscle (Wright, 46; Meyerhof, Gemmill, Benetato, 41). The initial heat production of the poisoned muscle is also similar to a normal muscle (Fischer, 29; Hartree, 32; and Hill, 35). The respiratory quotient of resting frog muscle poisoned with iodoacetate is 0.91 (Saslow, 45). The addition of lactate to the muscle increases the oxygen consumption and respiratory quotient (Meyerhof and Boyland, 40). Glucose disappears from the surrounding medium if oxygen is present (Barker and Shorr, 25). A poisoned muscle in oxygen can carry out slightly more work than under anerobic conditions (Lundsgaard, 38). The mechanical and electrical responses of the poisoned muscle are not altered (Henriques and Lundsgaard, 34). These experiments provide sufficient evidence that the contractile process is not fundamentally changed by monoiodoacetate and demonstrate that the oxidative processes are not as sensitive as the glycolytic mechanisms. However, since the oxygen uptake of the triosephosphate system in the glycolytic scheme presented above is inhibited by monoiodoacetate and since the oxygen consumption of poisoned muscles during rest and activity is not greatly altered, an assumption may be made that under these conditions there are other methods for the utilization of carbohydrate in muscle than the way which involves the triosephosphates.

Mawson (39) made the interesting observation that a poisoned muscle furnished with lactate in oxygenated solutions carried out more work than one without lactate. Also, the poisoned muscle with lactate contained more phosphocreatine. Since the lactate disappeared these results indicate that an oxidative mechanism involving lactate and molecular oxygen is associated with phosphorylation. Grimlund (32) has extended these observations and found that lactate, pyruvate, succinate, glycerophosphate and fumarate had similar activity. The association of lactate oxidation with phosphorylation has not been shown, as yet, in muscle extracts.

Oxidizing agents. Hahn and his co-workers (51, 52) demonstrated that muscle *brei* incubated for two or three hours at 38° C. in absence of oxygen produced more lactate than when oxygen was present in a similar mixture. Later, they (53) obtained a substance that was free of protein and was precipitated by barium which inhibited lactate formation when added to muscle *brei* in

oxygen. These authors believed that they were dealing with a true case of inhibition and not a coupling of lactate formation and subsequent oxidation when oxygen was present. In contrast to these findings of Hahn, Meyerhof (59) claimed that glycolytic activity of cell-free extracts was independent of oxygen. Also, Lipmann (56) stated that the glycolytic enzyme was not autoxidizable and muscle extracts which do not contain respiratory ferments glycolyze in oxygen as well as in nitrogen. Their evidence, therefore, was against the direct action of oxygen in glycolysis when the respiratory mechanisms were absent. Nevertheless it would be interesting to determine if the substance isolated by Hahn was acting as an oxidizing agent, for Lipmann (56) showed that glycolysis was inhibited by the addition of iodine and quinone to muscle extracts. This author also demonstrated that when the indophenol system was added to an oxygenated muscle extract, glycolysis was inhibited and this inhibition was removed by the addition of ascorbic acid and replacement of the oxygen by nitrogen. Thus when the natural respiratory ferments were replaced by the indophenol system the glycolytic enzymes were inhibited. Lipmann's conclusion was that the oxidized form of the glycolytic enzyme was inactive while reduction allowed its glycolytic activity to take place. Gemmill and Hellerman (50) confirmed the observation of Lipmann that iodine was an inhibitor and showed that this inhibition was removed by the addition of reducing agents as cysteine, glutathione or ascorbic acid. Geiger (48) demonstrated that oxidized glutathione may inhibit glycolysis in muscle *brei*. Rapkine (64) showed that the enzymatic reaction triosephosphate + pyruvate $\rightleftharpoons$ phosphoglycerate + lactate was sensitive to oxidized glutathione or iodine and that this inhibition was reversed by hydrogen sulfide, reduced glutathione or cysteine. Rapkine also showed that the effect of oxidants and reductants was on the enzyme itself and not on the coenzyme. Leöwey (55) demonstrated that the conversion of pyruvate to lactate was inhibited by iodine and quinone and this inhibition was reversed by hydrogen sulfide.

The inhibitory action of oxygen and of oxidizing agents on muscle enzyme systems have their application to a fundamental problem in muscle physiology. This problem is whether the production of lactate is the essential reaction and the oxidative removal of lactate a secondary process or whether the direct oxidation of carbohydrate is the fundamental process in supplying the energy for muscular contraction. It would be impossible at the present time to give all the evidence for or against these ideas. The majority of workers in this field consider that, under aerobic conditions, oxidative changes occur in place of the lactate formation. Meyerhof (60), in 1927, stated that the splitting of sugar to lactate and the oxidation were two distinct processes and the formation of lactate was not to be regarded as the introductory stage in carbohydrate combustion. Sacks and Sacks (65) maintained that oxidative changes involving molecular oxygen supplied the chemical energy for muscular work. Dill and his associates (58) divided the recovery period in man into a lactacid and an alactacid phase and claimed that the lactate mechanism would not play any important part in muscular contraction except in very strenuous exercise. Gemmill (49) pointed out that anaerobic metabolism occurs in the intact animal but only as a reverse mechanism, that the aerobic form is normally present, and that the aerobic metabolism of carbohydrate would be an entirely different process from the anaerobic metabolism. Bugnard (47), working in Hill's laboratory, reported that there was a possibility that a normal muscle giving a single twitch or a series of twitches at a very low frequency contracted without the formation of lactate and the latter mechanism came into play only when oxygen want was impaired. Meyerhof (61) discussed the glycolytic systems as the "Chemistry of the Anaerobic Recovery of Muscle". Needham, in a recent review (62), stated that the oxygen utilization in muscle took place so rapidly that it was not used for removal of lactate or resynthesis of phosphocreatine and she suggested that it might be used directly by the myosin fibrils. Lundsgaard (57), in a recent address, said that the formation of lactate was not an essential element in the chemistry of muscular contraction, nor was intermediate lactate formation a necessary element in the production of mechanical energy from oxidations in the muscle. Therefore a large number of workers in the field of chemistry of muscular contraction agree that there is a separation of aerobic and anaerobic breakdown of carbohydrate in muscular contraction, but disagree as to the position of the short circuit. If Lipmann's theory is correct that the oxidized form of a glycolytic enzyme is inactive and if this enzyme is kept in the inactive oxidized state by the respiratory ferments, then no part of the glycolytic system presented in the first section may play a role in carbohydrate oxidation under aerobic conditions, as the first oxidation in this system involves the triosephosphate dehydrogenase. However, Lipmann's idea that the normal respiratory ferments keep a system in an oxidized form when oxygen is present has another explanation. It may be that the triosephosphate is oxidized to phosphoglycerate by the coenzyme + coenzyme factor + cytochrome system. Needham and Lu

(63) showed that such a system took up molecular oxygen. This idea would demand that this system be able to compete with the pyruvate-lactate system for the reoxidation of the reduced coenzyme and the phosphoglycerate, in turn, be converted to pyruvate which would be oxidized. Szent-Györgyi (66) suggested that the system oxaloacetate + malate + furmate + succinate worked at this point. Krebs' (54) citrate cycle might also be used in this oxidation. Although there are numerous pathways for the oxidation at the triose stage there may be many other methods for oxidizing the carbohydrate molecule *in vivo* which do not involve triosephosphate formation and these are able either to supply energy for phosphorylation or to transfer energy directly to the contractile elements in the muscular machine.

Heavy metals. Cotzhausen and Schallehn (67) made a systematic survey of the action of heavy metals on the breakdown of glycogen to reducing sugars in muscle extracts. They found that reducing sugars accumulated in the presence of cupric and ferric salts in concentrations of 10^{-4} to 10^{-9} mols. Cobalt, manganese, zinc, silver and mercury salts had no effect on this reaction. These results could be interpreted either as an accumulation of a reducing sugar due to inhibition of its breakdown or to an acceleration of amylase activity. Lipmann (71) studied the action of cupric sulfate on glycolytic activity of muscle extract and found that 9×10^{-5} molar solutions of cupric ion increased glycolytic activity and 4.5×10^{-4} molar solutions inhibited glycolysis, while ferrous ion in any concentration produced only inhibition. These reactions were independent of oxygen. Wagner-Jauregg and Rzeppa (76) showed that cupric sulfate inhibited glycolysis, ferrous ion had a slight effect, and ferric, zinc and cadmium ions had no effect. This heavy metal inhibition was removed by the addition of coenzymes I or II. Gemmill and Hellerman (68) using phenylmercuric hydroxide, *p*-chloromercuric benzoate and mercuric chloride, demonstrated that these substances caused inhibition. The inhibitory effect was removed by cysteine or glutathione and the degree of inhibition depended on the concentration of the mercury compound. The inhibitory action was on some system involving the hexosephosphates or triosephosphates, as glycogen disappeared from the mercury-containing extracts but not "total carbohydrate". Rapkine (72) recently demonstrated that the triosephosphate dehydrogenase was inactivated by cuprous oxide and restored by hydrogen sulfide. Gill and Lehmann (69) have shown that zinc ions inhibit phorphorylation.

Since oxidizing agents, monoiodoacetate and heavy metals can react with sulfhydryl groups, the activity of these three types of inhibitors may be on such a group in an enzyme or enzymes in the glycolytic system. Such a possibility has been discussed by Gemmill and Hellerman (68) and by Rapkine (72) for glycolysis, and by Hellerman (70) for general enzymatic catalysis. It is interesting to note that Thunberg (75) in 1911 suggested the possibility of sulfhydryl groups reacting with poisons in the animal body. This theory, however, does not meet all the known facts concerning the three classes of inhibitors. For example, Stannard (73) has demonstrated that iodoacetamide inhibits glycolysis of muscle extracts less readily than iodoacetate. This observation was in the reverse order of the velocity of the reaction with sulfhydryl groups in compounds of known composition (Smythe, 74). The component enzymes of the glycolytic systems have not been studied extensively enough to base any conclusions as to the validity of the idea that inhibitory activity of monoiodoacetate, oxidizing agents and heavy metals can be related to the sulfhydryl group in one enzyme. Nevertheless Rapkine's (72) work suggests that the triosephosphate dehydrogenase may be of special importance in this connection as it is inhibited by monoiodacetate, oxidized glutathione and cuprous oxide. More work is needed before this interesting idea can be confirmed or permanently rejected.

Glyceraldehyde. Following the discovery of Mendel (82) that glyceraldehyde inhibited the anaerobic glycolysis of tumor cells, numerous attempts were made to apply this discovery to muscle extracts. Adler, Calvet and Günther (77) obtained inhibition of glycolysis when glycogen was used as substrate but not with hexosemonophosphate. This indicated that the inhibition was on the primary phosphorylating mechanism. Boyland and Boyland (79) also showed that *dl*-glyceraldehyde inhibited glycogen breakdown and esterification. In contrast to these experiments are those of Holmes (80) and of Baker (78). Both claimed that glyceraldehyde had no effect on the glycolysis of starch in muscle extracts. Lehmann and Needham (81) explained these dissimilar results on the basis that, in fresh glyceraldehyde solutions, this substance was in the dimeric form and, in that case, had an inhibitory action similar to glucose on the formation of hemosemonophosphate from glycogen. In old solutions, where this compound was in the monomeric form, phosphorylation was not inhibited while the non-phosphorylating breakdown of glucose was stopped. Süllmann recently (83) repeated and extended these observations. This author reported that glyceraldehyde in relatively large concentrations inhibited glycolysis but not esterfication, and he placed the inhibitory effect

on an aldo-condensation between the triosephosphate and glyceraldehyde. In the light of these conflicting results glyceraldehdye inhibition cannot be used as a method for separating phosphorylating from non-phosphorylating mechanisms in muscle extracts.

Glucose. Haarmann (85) studied the lactate formation when different combinations of sugars were added to muscle extracts and found that the effect in some cases was not additive but inhibitory. Lehmann (86), in studying the velocity of hexosemonophosphate formation from inorganic phosphate and glycogen, observed that not only the end product of this reaction, hexosemonophosphate, but also glucose inhibited this reaction. Lehmann and Needham (87) compared this inhibition to that of glyceraldehyde and found that both require rather high concentrations to bring about the effect. Thus the reaction of glycogen with phosphate to form hexosemonophosphate can be inhibited by the addition of a non-phosphorylated carbohydrate. Cori, Colowick and Cori (84) demonstrated that the hexose-1-phosphate had a greater inhibitory activity on phosphorylation than the 6-ester; glucose in 17 mM concentration also inhibited phosphorylation, while fructose and maltose were without effect. These authors remark that the inhibitory effect of glucose probably does not play a physiological role, for even at a blood sugar level of 300 mg. per cent the glucose level in the tissues does not exceed 50 mg. per cent. At this concentration glucose has no inhibitory effect on phosphorylation in extracts. However, what this concentration of glucose may do in intact muscle cells has not been determined.

Phlorizin. Lundsgaard (94) demonstrated that phlorizin stopped lactate formation in muscle extracts. In fluoride-poisoned muscle *brei,* carbohydrate esterification and glycogen breakdown were also inhibited. Parnas and his associates (96) placed this inhibition on the formation of a hexosemonophosphate. Cori, Colowick and Cori (88) showed that the site of inhibition was on the formation of hexose-1-phosphate and not the transformation of the 1-ester to the 6-ester. Kalckar (92) described another site of inhibition; namely, the conversion of triosephosphate to phosphoglycerate. However, this reaction was not as sensitive as the initial phosphorylation, for Needham and Pillai (95) showed that while the esterification of glycogen was 80 per cent inhibited, lactate formation from hexosediphosphate was only 15 per cent inhibited by the same concentration of phlorizin. Both Lundsgaard and Cori remarked that the concentrations used in enzymatic solution were much greater than those reached in the intact animal. Therefore, these results do not have any physiological significance with regard to the metabolism of intact muscle and it is not surprising that a phlorizinized animal is able to use carbohydrate. Deuel, Wilson and Milhorat (89) showed that some of the glucose given to a phlorizinized animal was oxidized and Drury, Bergman and Greeley demonstrated that a phlorizinized dog after hepatectomy used sugar. During exercise the blood lactate increases in a phlorizinized dog (Loebel, Barr, Tolstoi and Himwich, 93) and the carbohydrate content in the muscles of a phlorizinized rat decreases (Gemmill, 91). However, it would be interesting to raise the concentration of phlorizin in an animal or in an isolated muscle to a level comparable to that used in muscle extracts in order to see if this agent might affect muscular contraction.

Insulin. In contrast to the large number of observations on the formation and storage of glycogen in animal tissues under the influence of insulin, only one claim has been made for insulin as an inhibitor in muscle extracts. Lehmann (98) stated that he was able to obtain inhibition of the breakdown of glycogen in muscle extracts with this agent. The point of inhibition was placed at the formation of the Embden ester. In a later publication Lehmann (99) continued this report and stated that M/20,000 insulin inhibited esterification and that the insulin effect was decreased by the addition of magnesium. However, Cori, Colowick and Cori (97) remarked that they were unable to confirm the results of Lehmann and, in addition, found that insulin had no effect on the formation of the 1-ester. More work is needed on this important subject if a definite enzymatic function is to be attributed to insulin action.

Sulfite. With sulfite, Case and Cook (101, 102) were able to demonstrate pyruvate formation in muscle, and Meyerhof and McEàchern (103) showed that the lactate production was reduced as the pyruvate formation was increased. The maximum inhibition of lactate production was only about 50 per cent. Later, Meyerhof and Kiessling (103) used sulfite for demonstrating that not only pyruvate was formed in muscle extracts, but also glycerophosphate. This inhibition of glycolysis is produced by combination of the inhibitor with an intermediate product in the glycolytic system and not by enzymatic inactivation.

Snake Venoms. Chain (104) reported that certain snake venoms inhibit glycolysis and that the inhibitory substance for a few species was thermostable. He also demonstrated (105) that the venom from the black tiger snake had a special inhibitory activity on coenzyme I. The action of this venom may be on the phosphate groups in the molecule of the coenzyme and thus it would be classified as a nucleotidase. Whether all snake venoms have similar activity is not known, but it would explain the activity of this venom on glycolysis through destruction of the coenzyme.

Oxalate and Citrate. Lohmann (106) observed that the inhibitory effect of oxalate and citrate on the glycolytic activity of muscle extracts was very similar to fluoride activity as phosphorylation was stopped and the same ester which was difficult to hydrolyze accumulated in these solutions. Later studies demonstrated that the ester was phosphoglycerate. Lohmann and Meyerhof (107) showed that the action of oxalate was on the breakdown of phosphopyruvate to pyruvate and phosphate while citrate, in strong concentrations, inhibited the transformation of phosphoglycerate to phosphopyruvate as well as the dephosphorylation of phosphopyruvate.

The question of inhibition of glycolysis has many practical applications not only in the physiology of muscular activity but also in pathological conditions. Why does a muscle from an adrenalectomized animal have impaired lactate formation (Buell, Strauss, Andrus, 108)? How does insulin promote storage of glycogen in muscle? How is glycolysis in muscle affected by vitamin deficiencies? These are some of the questions which may be answered by the application to the physiological inhibitors of the same methods as have been used with the unnatural agents described in this paper.

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DISCUSSION

Dr. Barker: I might start out by bringing up the problem that some of us who work on intact tissues encounter when we try to apply these so-called specific poisons to the study of the respiration of intact tissues. For instance, it has been stated that iodoacetic acid specifically blocks the enzymatic dismutation of triosephosphate; therefore, the addition of lactate to an iodacetate-treated tissue should enable the tissue to maintain its oxygen consumption up to normal. That is true for a while, but even if one takes the lowest concentration of iodoacetate which will stop anaerobic glycolysis, presumably through its specific effect, the lactate effect on oxygen consumption does not keep up, and apparently there are other inhibitory processes which take place. Our experience with fluoride has been somewhat the same. Although one reduces the concentration of these materials to as low a point as is compatible with the inhibition of the particular process under examination, one still gets an effect on other processes which is reflected in a decreased oxygen consumption. It may be that a disturbance in cellular organization takes place which cannot be studied. Our experience with organic mercury compounds has been somewhat the opposite, the highest concentrations we were able to get in solution have practically no effect on oxygen consumption. The solubility and penetration into the cell are limiting factors there. Another interesting effect was obtained with phlorizin. Some time ago, Shorr, with Richardson and Loebel, studied the effect of the oxidation of glucose by various tissues of phlorizinized rats, and found there was good oxidation of carbohydrate. When phlorizin was added *in vitro* to the isolated tissue, high concentrations of the drug had to be reached before there was any effect on oxygen consumption.

The most recent claim as to the functioning of insulin, that of Krebs, is quite out of line with the usual effect of insulin on carbohydrate storage. The *in vitro* increase in oxygen consumption, and presumably in carbohydrate oxidation, had not been found in intact cells, nor in the whole animal.

Dr. Gemmill: Barker's remarks illustrate the difficulty in applying these inhibitors to the intact cells. We have fairly accurate evidence that in the glycolytic system the action of an inhibitor is

on a particular enzyme, and it remains for future work to determine whether the same enzyme is attacked in an intact cell.

Dr. Barker: There certainly is a considerable effect of cellular organization on what takes place in tissue metabolism. We found, for instance, if we studied intact skeletal muscle tissue from a fasted animal, that it had an R.Q. of 0.75, indicating fat oxidation, whereas if we minced it, we found it was oxidizing carbohydrate almost exclusively. A complete change in metabolism followed disruption of cellular organization.

Dr. Burk: I do not believe that I heard you mention any possibility of calcium being involved. The possibility of fluoride, citrate, or oxalate affecting calcium is well known in many other systems. We have observed a simple quantitative correlation between the action of fluoride and oxalate in relation to the solubilities of their calcium compounds. The inhibition by fluoride or oxalate in respiration and growth of *Azotobacter,* which requires calcium in both processes, yields relations which one would expect merely on a basis of relative solubilities, the oxalate being several times more inhibitory, and its calcium compound being several times more insoluble than in the case of fluoride.

Dr. Gemmill: I do not know anything about the role of calcium in the glycolytic systems but one would think that if fluoride was combining with an inorganic component it would combine with magnesium and inhibit phosphorylation. However, it does not have any effect on the mechanisms of phosphorylation, and therefore the combination cannot be with this specific inorganic component.

Dr. Hellerman: I would like to ask about the occurrence and apparent function of intracellular amylase. I have rarely heard this discussed in connection with glycolysis. The question seems especially pertinent in view of the beautiful demonstration by Cori and Cori of phosphorylytic splitting of glycogen in various tissues.

Dr. Gemmill: It is not known, at the present time, just what role amylase plays in the muscle. In fact, we believe that the contracting muscle cannot use glucose as such, but uses glycogen. Therefore, there would be no need to break down the glycogen into glucose. There is no room in this present glycolytic system for amylase; in fact, it is an inhibitor.

Dr. Burk: I would like to go on record in a loud voice against the statement that there is no advantage to the old lactic acid theory. It seems to me it is just about as good as it always was, and that all we have done is to go beyond it. It is now known that lactic acid is one reservoir which is a smaller reservoir than the energy producing reservoir represented by oxygen, and that there are still smaller reservoirs which, when the

lactic acid reservoir is cut out, then empty themselves. And, while it may be true that under some conditions the lactic acid reservoir is not called upon, it seems to me that the old Meyerhof-Hill concept is still very good indeed, for where it was originally applied. I think for the present we must still continue to regard the lactic acid reservoir as normally operating even if it can be turned off sometimes.

Dr. Gemmill: What evidence do you have that under aerobic conditions lactate is formed?

Dr. Burk: First there is no clear evidence against it. Since there is inferential evidence for it from all sides, I think that, for the present point of view, we should favor it.

Dr. Gemmill: The evidence for lactate production has been obtained by artificial subdivision. By experimental methods a muscle is stimulated in nitrogen and lactate is formed; it is then stimulated in oxygen and oxygen is used and carbon dioxide is given off. The assumption is made that under aerobic conditions the same initial processes are taking place as under anaerobic conditions. Another assumption may be made that there is a short circuit before the lactate formation. We have not definite evidence for or against the two ideas.

Dr. Hellerman: There is no evidence, however, that glycogen cannot be resynthetized from lactate.

Dr. Gemmill: The evidence is conclusive for frog muscle but not so definite for mammalian muscle. It may be that it is necessary to have the lactate carried to the liver for the complete cycle.

Dr. Barker: I would like to ask Burk what tissues besides muscle this lactate reservoir would supply? Kidney forms very little lactate under any condition.

Dr. Burk: I did not quite mean, earlier, to distinguish between going all the way to lactate and going most of the way, as for instance to pyruvic. The formation of lactate anaerobically calls for the largest amount of energy, of course, but pyruvate formation would yield not far from the same.

Dr. Gemmill: I think we are in agreement on that. When you spoke of a lactate reservoir you meant the energy which could be derived from any part of that system.

Dr. Burk: Yes.

Dr. Barker: I think that much depends on how far you can separate the aerobic and anaerobic processes. If you study glycolysis manometrically, the only thing you know is that no acid material is found, so that you can eliminate lactic acid and pyruvate definitely. How far back beyond that you go would depend entirely upon what specific chemical analyses you perform.

Dr. Burk: I think I can state my conception of the Meyerhof-Hill theory a little more fully.

While it is true that in certain aerobic conditions one gets no lactic acid, whereas in anaerobic conditions one does, nevertheless there exists all intermediate varying amounts as one goes from complete anaerobiosis to complete aerobiosis. I may cite the work of the Harvard group, Hill, and others, who show that as the muscular activity greatly increases the state of anaerobiosis increases and there are very definite and large amounts of lactic acid formed; in thirty seconds of intense running there are extremely large amounts present, fatigue is temporarily almost complete, and a period of an hour or more is needed for blood lactic acid to return to normal. We can certainly call this a fairly normal case.

Dr. Gemmill: It is only after strenuous muscular exercise that lactate accumulates in the blood. It appears that it is a reserve mechanism which enables us to carry out exercise at a very rapid rate.

Dr. Burk: You call it a reserve mechanism; but it is just the same as I considered it before—as a reservoir.

Dr. Baumberger: In the normal functioning of the muscle *in situ,* there may be periods of anaerobiosis and aerobiosis respectively, during the contraction and relaxation of the muscle. There is a diminished flow of blood through the muscle during sustained contraction at that time. There may possibly be a consumption of all or part of the oxygen bound by the myohemoglobin which might lead to the anaerobic phase of the metabolism forming lactic acid. When the muscle relaxes and blood again flows, the lactate might be completely oxidized before it gets into the blood. And therefore, even in slow exercise, you might find no lactate in the blood, and yet it might have been formed in the anaerobic period.

Dr. Gemmill: Hill calculated that there was sufficient oxygen in an isolated muscle for five or six contractions, and therefore there is a sufficient amount of oxygen present to carry out a small amount of work before the anaerobic phase.

Dr. Baumberger: Most contractions in the intact organism are tetanic in nature and during a tetanic contraction blood flow is much diminished.

At what particular oxygen tension is the triosephosphate dehydrogenase inhibited?

Dr. Gemmill: It has not been worked out. There is the competition between that system and the pyruvate-lactate system. It would be interesting to find out when the pyruvate-lactate system would come into play in contrast to the cytochrome-oxygen system.

FUMARATE IN BIOLOGICAL OXIDATIONS*

Fredrick J. Stare and Carl A. Baumann

The living cell is an extremely dynamic entity, its constituents being in a constant state of flux—constantly in the process of synthesis and degradation. The cell lives by virtue of a highly complex equilibrium between a large number of reactive chemical compounds. One of these compounds is fumarate. A discussion of fumarate in cellular respiration, therefore, involves a consideration of many substances other than fumarate, since fumarate is biologically interesting only because it is readily transformed into other substances, and because other substances are readily transformed into it.

The complexity of the respiratory process has led to much speculation as to the chemical mechanism involved, and the theories which have been formulated are both numerous and contradictory. Unfortunately, the theories have sometimes received more attention than the facts on which they were based. Theories have, however, served as convenient pegs on which to hang an assortment of isolated facts. Two distinct theories have arisen about the action of fumarate and related substances; the Szent-Györgyi theory concerning hydrogen transport, and the Krebs citric acid cycle dealing with the breakdown of carbohydrate fragments.
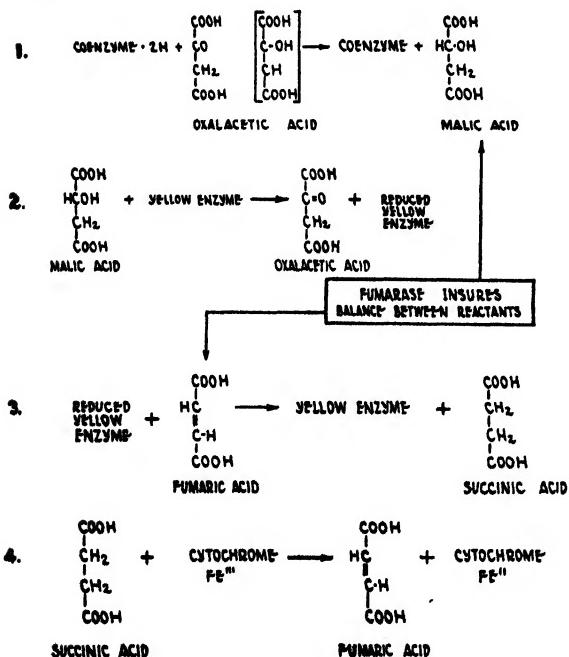
Szent-Györgyi's theory has undergone several modifications. The present version (1) is as follows: Substrate hydrogen is transported from reduced coenzyme to oxalacetic acid which is thereby reduced to malate. The malate reacts with yellow enzyme, reducing it, and the reduced yellow enzyme then passes the hydrogen to fumarate, reducing it to succinate. According to Szent-Györgyi it is succinate, and succinate only, which reacts with cytochrome. Schematically the theory can be represented as shown in Fig. 1. The real function of fumarate and its relatives, malate, succinate, and oxalacetate, Szent-Györgyi's "C_4", is that of hydrogen transport. The compounds are readily and rapidly transformed into one another, and hence only a small amount of "C_4" is needed to transport hydrogen from reduced coenzyme to cytochrome. Szent-Györgyi actually considers "C_4" to be the active prosthetic group of the hydrogen-carrying enzymes, the "transportases."

In the Krebs citric acid cycle (2) the "C_4 acids" are assumed to function in the degradation of carbohydrate. The cycle can be pictured as shown in Fig. 2. Oxalacetate condenses with a
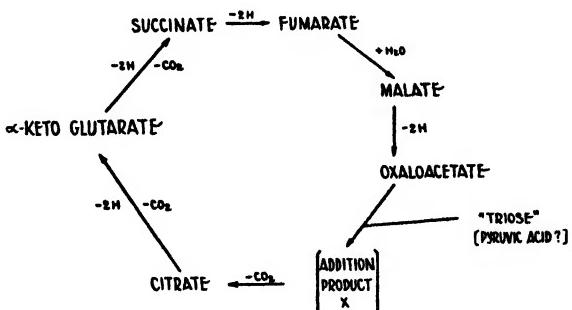
Fig. 2. The citrate cycle of Krebs.

"triose" or its oxidation product to form an intermediate which loses CO_2 and hydrogen, forming citric acid. The citric acid rearranges, loses CO_2 and hydrogen and eventually forms a keto-glutaric acid, which also loses CO_2 and hydrogen, yielding succinic acid which is transformed successively to fumaric acid, malic acid, and finally oxalacetic acid completing the cycle. As a picture the Krebs cycle has the virtue that it offers a comprehensible pathway by which CO_2 is formed by the cell, and furthermore, the step-wise formation of CO_2 harmonizes with the step-wise removal and transport of hydrogen.

The purpose of the present discussion is to consider some of the available experimental evidence dealing with fumarate and related sub-

Fig. 1. Hydrogen transport according to Szent-Györgyi.

stances, which has given rise to the above theories.

The first evidence to be cited in connection with the above theories concerns succinodehydrogenase, the enzyme which converts succinate to fumarate. This enzyme is extremely widespread and extremely active (3, 4). It has been found in all animal tissues as well as in bacteria and yeasts. This enzyme differs from most dehydrogenases in several important particulars. It not only is one of the most active respiratory enzymes known, but the enzyme belongs to the "zero order," that is, maximum activity is observed with very small concentrations of substrate (5). This property enables tissues to convert very small amounts of succinate very rapidly.

When moderate amounts of succinate[1] are added to minced tissue the rate of oxygen uptake is increased enormously. The rate of respiration, however, in contrast to oxygen consumption, is not correspondingly increased. Effectively, what happens is that the dehydrogenation of succinate is superimposed on normal respiration. This can readily be demonstrated by considering the respiratory quotient, which is lowered markedly by succinate. If the volume of oxygen consumed in normal respiration be designated as X, the CO_2 output will also be X, since the R.Q. of normal muscle tissue is approximately 1.0. On the addition of succinate the oxygen consumption may be doubled or more. Assuming it to be doubled, the extra oxygen consumption due to succinate will also be equal to X. There will, however, be no additional production of CO_2, since the enzyme merely transfers hydrogen from the substrate. The hydrogen ultimately appears as water; the end-product of the carbonaceous residue is fumarate. The calculated R.Q. will be $X/(X + X)$ or 0.5. In actual experiment, amounts of succinate have been added such that the oxygen uptake was more than doubled. Under these conditions R.Q. values of 0.43 have been observed (6). These experiments are important because they show that normal tissue contains a mechanism which can mobilize much larger amounts of hydrogen from succinate than the amounts of hydrogen ordinarily mobilized from the natural substrates in normal respiration. Kinetic studies likewise emphasize the enormous hydrogen-carrying capacity of succinate. Szent-Györgyi and coworkers have shown that the small amounts of succinate present in normal tissue are oxidized at a sufficiently rapid rate to account for all of the hydrogen transported to cytochrome in normal respiration (7, 8).

A second peculiarity of the succinate-succino-

dehydrogenase system is its unique ability to react with cytochrome. In Keilin's experiments on the hydrogen-carrying function of cytochrome (9), succinate was employed as the substrate, which was able to reduce cytochrome in the presence of succino-dehydrogenase. A survey of other dehydrogenase systems, however, indicated that they do not possess this property. Ogston and Green (10, 11) examined 11 different dehydrogenase systems, and observed reduction of cytochrome only in the presence of succino-dehydrogenase. Szent-Györgyi and co-workers (1) likewise emphasize the specificity of the reaction between succinate and cytochrome.

The salient facts about succino-dehydrogenase are the following: the system is extremely widespread in animal tissues, the system is extremely active, and the reaction with cytochrome is specific. It is therefore highly probable that the system is biologically important, despite the fact that the amounts of succinate formed in metabolic processes are relatively small, or despite the fact that no one has succeeded in demonstrating that succinate is a metabolite in higher animals. Szent-Györgyi provisionally assumed that the function of the succinate system was to transport hydrogen to cytochrome (7).

Certain observations with inhibitors, however, tended to shift the emphasis from succinate to fumarate. Thus malonate inhibited the action of succino-dehydrogenase (12), and malonate also inhibited respiration (7, 8). The addition of succinate to a system poisoned by malonate did not appreciably increase respiration. However, the addition of fumarate completely compensated for malonate inhibition (6, 8). Not only that, but fumarate actually increased respiration in the presence of malonate, the increase being for all practical purposes the same as when malonate was absent (Fig. 3).

The increase in respiration due to fumarate was in reality a preservation of the initial rate, rather than a true increase. Banga (13) reported that when fumarate was added to pigeon breast muscle suspended in M/15 phosphate at pH 7.4, the initial rate of respiration was maintained for 30 minutes or more, whereas similar tissues showed a rapidly decreasing rate of respiration in the absence of added fumarate. This observation has been confirmed repeatedly (6, 14, 15). Since the function of the added fumarate appeared to be to conserve the respiration rather than to increase it, the assumption was made that necessary fumarate diffused out of the cells into the surrounding medium, decreasing the concentration within the cell until fumarate became the limiting factor in respiration. As the amount of fumarate which preserved respiration, 0.0066 M, roughly approximated the amount present in the tissue, there

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[1] When very small amounts of succinate are added to respiring muscle, a true catalytic effect is observed. See below.
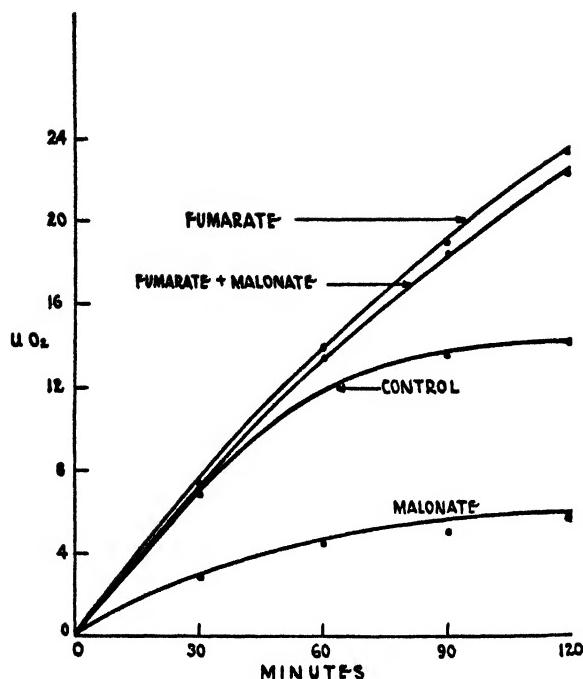
Fig. 3. The effect of malonate on the respiration of pigeon breast muscle stimulated by fumarate. 0.005 M fumarate and malonate. The results are expressed in terms of oxygen uptake (U_{O_2}), defined as the mm.³ of oxygen absorbed per mg. dry weight of tissue in the time t (6).

appeared to be some validity to the assumption. Szent-Györgyi therefore postulated that it was not the succinate-fumarate which functioned in hydrogen transport, but rather fumarate-oxalacetate (8). Unfortunately the "preserving" effect of fumarate is not generally observed under the experimental conditions originally outlined by Szent-Györgyi. Of 24 experiments with pigeon muscle in phosphate, we observed a true conserving effect only 7 times. With 21 pig hearts, we never observed a true conserving effect, although fumarate almost always increased respiration somewhat (6). Furthermore, the amount of fumarate necessary to increase respiration in this medium was so great that the increased oxygen consumption could have been due entirely to the oxidation of the fumarate—that is, the fumarate might have been acting only as a substrate. As most tissues contain an extremely active fumarase which converts fumarate to malate, and as they also contain a malico-dehydrogenase, the substrate action of fumarate might be explained on this basis. However, as far as could be determined by questionable chemical methods of analysis, the amount of fumarate plus malate did not decrease during respiration (8, 16).

A study of the respiratory quotient indicated that the respiration of tissue fortified or preserved

with fumarate was practically normal—in other words, that the measured oxygen uptake represented true respiration. We have observed R.Q. values of approximately 1.0 for fumarate respiration with both pig heart and pigeon breast muscle, using several media: M/15 phosphate, Ringer-phosphate, or the two media fortified with muscle juice (6). Szent-Györgyi reports that the respiratory quotient falls from 1.0 to approximately 0.8 as the respiration of muscle decreases (1, 17). When respiration is maintained by means of fumarate, the value of the respiratory quotient is maintained at the normal value, 1.0.

Proof of the catalytic action of fumarate came when very small amounts of fumarate were added to minced pigeon breast muscle suspended in Ringer-phosphate solution (6). In this medium much smaller amounts of fumarate conserved respiration than in plain phosphate buffer, 0.0002 M being approximately equivalent to 0.0066 M in the latter medium. A calculation of the added oxygen consumed in the presence of fumarate revealed that much more extra oxygen was consumed than could be accounted for by the complete oxidation of the fumarate to CO_2 and water. For example, in 20 experiments with concentrations of fumarate from 0.0001 to 0.0008 M, all showed increases in oxygen consumption much greater than could have been consumed by the fumarate. The discrepancy between the theoretical and the observed increases in oxygen consumption was sometimes quite spectacular. For example, when sufficient fumarate was added to form a 0.0002 M solution, the amount of oxygen sufficient to completely oxidize this fumarate in 3.00 cc. was 40 mm.³ The increase in oxygen consumption actually observed with this amount of fumarate was 255 mm.³, an increase over the theoretical of 6-fold.

More recent experiments indicate that the discrepancy may persist, that is, fumarate may act catalytically in concentrations up to 0.002 M. There is, no doubt, some catalytic action at even higher concentrations, but at these higher concentrations a substrate effect is also operative. Not only fumarate, but substances which yield fumarate in the presence of respiring tissue increase the respiration of pigeon breast muscle in an analogous manner. Low concentrations of succinate, malate, oxalacetate, or citrate all produced increments in oxygen consumption greater than could be accounted for by the complete oxidation of the material added. Table I presents recent data illustrating the catalytic activity.[2]

[2] Unless otherwise stated all of our experimental work discussed in this paper has been done using Ringer-phosphate buffer (6) (PO_4 = M/60, pH = 7.4, 0.2 p.c. glucose).

TABLE I

The increase in the respiration of pigeon breast muscle stimulated by small amounts of fumarate and allied substances.

Concentrations	Oxygen uptake in mm.*	
	Observed increase*	Theoretical increase from complete oxidation (6)
0.0002 M fumarate	197	26.8
0.0004 M "	169	53.6
0.001 M "	298	134.0
0.002 M "	308	269.0
0.0004 M succinate	137	62.8
0.0004 M malate	134	53.6
0.0002 M citrate	69	40.3
0.0004 M "	126	80.6
0.001 M "	211	202.0
0.002 M "	115	403.0

* Increase calculated as follows: $U_{O_2}^{120''}$ of experimental sample *minus* $U_{O_2}^{120''}$ of control sample *times* dry weight of experimental tissue.

The final evidence of catalytic action was a demonstration of catalytic potency of the medium in which tissues had respired (6). The experiments were performed as follows: small amounts of fumarate, 0.0004 - 0.0008 M, were added to pigeon breast muscle and the muscle allowed to respire for 2 hours, during which time an increased oxygen uptake was observed with respect to control muscle. At the end of the period, the tissue was filtered off and aliquots of the fluid or "used juice" were added to fresh respiring pigeon breast muscle. "Used juice" from control experiments, unsupplemented with fumarate, showed a slight inhibitory effect on the respiration of fresh tissue. "Used juice" from experiments in which the fumarate has been added originally, still stimulated respiration somewhat; it showed a marked stimulation when compared to the "used juice" from the control samples. The catalytic potency of the fluid containing small amounts of fumarate had therefore been retained, and this in spite of the fact that the respiration in the original flask had been increased to a much greater extent than would have been sufficient to destroy all of the added fumarate.

It was thus established that fumarate can act catalytically *in vitro* under certain special circumstances. Although the critical experiments had been performed with pigeon breast muscle, other tissues were subsequently shown to behave in a similar manner, usually qualitatively and sometimes also quantitatively. The advantages of pigeon breast muscle are its comparatively high rate of respiration, the ease and speed with which large amounts of tissue can be obtained from a single animal, and the low cost. It is highly desirable that the experiments begin as soon after removal from the animal as possible. The pigeon is beheaded, the breast muscle removed and placed on ice for 2 or 3 minutes, and then minced in a chilled Latapie mincer, the muscle being collected in a chilled petri dish containing filter paper moistened with saline solution. For accurate work the tissue samples must be weighed separately. Attempts at pipetting suspensions of Latapie mince, even with the use of a wide-mouthed pipette, gave variable results. We weigh our tissue samples on a torsion balance, on small pieces of cellophane, which are added to the reaction flask with the tissue, and the tissue dispersed by means of a wire. Twenty to twenty-five minutes elapse from the time the bird is beheaded until the zero reading.

With some tissues it is important that a fairly constant amount of tissue be used for a given volume of medium, since the rate of respiration decreases if the amount of tissue is too low—Krebs' "dilution effect" (18). For pigeon breast muscle in Ringer-phosphate medium this decrease is usually observed when the amount of tissue in 2 cc. of medium is 100 mg. fresh weight or less. In most of our experiments we used approximately 150 mg. of tissue. Supplements such as *kochsaft* (deproteinized muscle juice) or the "C₄ — C₆" acids abolish the dilution effect. In lower respiring tissues, such as rat skeletal muscle, the

TABLE II

The effect of dilution on the respiration of rat skeletal muscle.

Supplements	Oxygen uptake in U_{O_2}			
	Tissue = 100 mg.*		Tissue = 300 mg.*	
	1st hr.	2nd hr.	1st hr.	2nd hr.
0—Control	1.7	1.8	3.6	4.0
0.0004 M fumarate	2.4	2.9	3.4	3.7
0.0004 M citrate	1.7	1.9	3.0	3.4
0.0004 M citrate + 0.2 cc. *kochsaft*	5.3	8.0	5.4	7.4

* Wet weight.

dilution effect is more pronounced (Table II).

The difference between results obtained with minced tissue as compared to tissue slices might also be interpreted as a variation of the dilution effect, if one considers that there will be a greater diffusion of necessary substances into the medium when there is a greater degree of tissue destruction. However, cellular integrity is not essential for a demonstration of the fumarate effect. Tissues ground with sand do not respond to fumarate, but when *kochsaft* is added, the response is equal to that of intact tissue (6). Banga and Szent-Györgyi (19, 20) and Greville (21) have studied the respiration of tissue dispersions—particles which pass through muslin but which are removed by centrifugation—and find that they also respond to fumarate when suitable supplements are added.

Greville (15) working with muscle, and Elliot and Elliot (22) working with liver dispersions, have both emphasized the importance of the tonicity of the solution in demonstrating "C₄ effects." Using plain phosphate buffer M/15, they report increases in respiration when NaCl is added to the medium. In effect, this amounts to conversion of the medium from plain phosphate to the Ringer-phosphate type of medium, in which both the rate of respiration, and the sensitivity to fumarate are known to be greater (6). Elliot reports that action of malate, oxalacetate, citrate, α-keto-glutarate, and pyruvate is completely dependent upon the chloride ion.

A number of muscles other than pigeon breast, have been studied for their response to fumarate. Muscle from pig heart gave essentially the same results as muscle from pigeon breast, although the response to fumarate was quantitatively somewhat less (6). Minced rabbit heart is sensitive to both fumarate and citrate in Ringer-phosphate buffer. Using concentrations of 0.0004 M we have observed catalytic increases in respira-

tion with these materials both in the presence and absence of *kochsaft*. The response to fumarate was usually somewhat greater than the response to citrate, and with some rabbit hearts the response to fumarate was also more consistent: that is, fumarate not only stimulated the respiration of the unsupplemented tissue, but also of tissue supplemented with *kochsaft* or insulin or mixtures of the two. Citrate did not always stimulate the supplemented tissues. The Q_{O_2} of our control rabbit hearts averaged 12.0. The protocols of a typical experiment are given in Table III.

Rabbit skeletal muscle had a lower rate of respiration than rabbit heart ($Q_{O_2} = 5.0$) and the tissue was also less sensitive to fumarate. Fumarate increased respiration in 2 out of 3 samples of muscle, but in only one case was the magnitude of the increase sufficient to indicate catalysis. A catalytic increase in respiration due to citrate was never observed, although respiration was increased somewhat in 2 out of 3 cases.

Rat skeletal muscle had an even lower rate of respiration (average $Q_{O_2} = 2.0$) and it was less responsive to fumarate and citrate than rabbit muscle. This tissue showed a pronounced dilution effect; that is, respiration decreased as the amount of tissue was "diluted" with medium (Table III). When the tissue was sufficiently dilute, 100 mg. per 2 cc., respiration was increased by 0.0004 M or 0.02 M fumarate, though not catalytically. 0.0004 M citrate was inactive; 0.02 M citrate actually decreased respiration. In contrast to pigeon breast muscle, rat skeletal muscle needed *kochsaft* as well as citrate or fumarate to overcome the dilution effect. In general, *kochsaft* was the most effective single agent in abolishing the dilution effect, particularly with low respiring tissues.

Rat diaphragm has been studied by Greville (15) who noted that fumarate increased respiration while malonate inhibited it. Sensitivity to

TABLE III

The effect of fumarate and citrate on the respiration of rabbit
heart muscle.

Supplements	Oxygen uptake in U_{O_2}		
	1st hr.	2nd hr.	3rd hr.
0—Control	12.9	17.3	18.6
0.0004 M fumarate	16.2	22.4	24.3
0.0004 M citrate	14.7	20.9	22.0
0.2 cc. *kochsaft*	13.4	20.3	22.8
Fumarate + *kochsaft*	17.3	25.8	27.4
Citrate + *kochsaft*	14.4	26.0	32.2

fumarate was increased appreciably by mincing the tissue with scissors. The conclusion appeared to be that a certain amount of cellular damage was necessary before a fumarate effect could be demonstrated (even our Ringer-phosphate solution is not strictly physiological) although damage such as that produced by Ca ions rendered the tissue insensitive.

Chicken breast muscle is another tissue with a relatively low rate of respiration in Ringer-phosphate solution ($Q_{O_2} = 2.7$). Fumarate, citrate, malate, and succinate in concentrations of 0.001 M all stimulated respiration somewhat, but the increase was not sufficiently great to indicate catalysis. With 0.0004 M fumarate, however, catalytic action was indicated. With this tissue also, high concentrations of citrate, 0.01 M, inhibited respiration, although similar amounts of fumarate, malate, and succinate stimulated it. The inhibition due to high citrate could be corrected by the addition of *kochsaft*. It is of interest that a decrease in respiration due to high citrate has been recorded by Krebs and Eggleston (18), who, however, did not stress the point. They used pigeon breast muscle in M/15 phosphate.

Normal human skeletal muscle has a low rate of respiration ($Q_{O_2} = 3.8$). It shows a pronounced dilution effect. It also responds favorably to succinate, and occasionally to citrate, although the latter may inhibit respiration if present in higher amounts. Failure of succinate oxidation by human muscle tissue has been found in various myopathies (23).

Several tissues other than muscle have been studied for fumarate, "C_4", catalysis. Szent-Györgyi and co-workers (8) report that fumarate stimulates and malonate inhibits the respiration of rabbit kidney mince. Stare (24) has shown that fumarate preserves the respiration of rabbit kidney and liver mince in a manner similar to that

observed in pigeon breast muscle. Fumarate compensated for malonate in this tissue, the response depending upon both the absolute and the relative amounts of the substances present.

It has been possible to demonstrate the catalytic action of a number of substances with rat kidney. Both slices and mince were used. In the presence of glucose, catalytic action was observed in 2 out of 4 samples of mince containing 0.0004 M fumarate, 1 out of 3 samples containing 0.0004 M malate, and 1 out of 1 sample containing 0.0004 M succinate. In no case (4 experiments) was catalysis observed with 0.0004 M citrate. Increased respiration was observed with all 4 acids when the concentration was increased to the point where a substrate effect was operative. With kidney slices, catalytic increases in respiration were observed when low concentrations of citrate, fumarate, or malate were added to Ringer-phosphate solution containing glucose. In the absence of added glucose, there was very little stimulation of respiration with either citrate, fumarate, or malate. This was true for both sliced kidney and kidney mince, and appeared to indicate that the acids in question increased respiration by stimulating the oxidation of carbohydrate. The demonstration of catalysis might, however, also have been somewhat dependent on the rate of respiration of the tissue. The Q_{O_2} of minced kidney was 8.0 in the absence of glucose, and 9.1 in its presence; for kidney slices, the Q_{O_2} averaged 11.0 in the absence of glucose, and 15.3 in its presence. In all cases kidney exhibited a more constant rate of respiration than muscle. As with muscle, respiration was usually somewhat better in the presence of fumarate than in the presence of citrate. The results of an experiment with kidney tissue are given in Table IV.

Rat liver, like rat kidney, was usually sensitive to the "C_4 acids." Concentrations of fumarate

TABLE IV

The effect of fumarate and citrate on the respiration of rat kidney mince and slices.

Supplements	Oxygen uptake in U_{O_2}					
	No glucose in buffer			0.2 p.c. glucose in buffer		
	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	3rd hr.
Mince						
Control	8.0	13.3	18.6	8.3	15.8	20.4
0.0004 M fumarate	8.2	13.5	17.7	9.6	19.0	25.1
0.0004 M citrate	7.5	12.9	17.7	8.8	15.9	21.3
Slices						
Control	11.0	18.6	26.1	13.6	27.2	39.3
0.0004 M fumarate	8.2	14.1	19.6	20.6	43.0	62.7
0.0004 M citrate	5.8	9.8	13.3	17.8	37.7	54.3

and citrate of 0.01 M and 0.001 M, increased respiration in 2 out of 3 samples of minced liver, and in 1 out of 3 samples of slices. Low concentrations, 0.0004 M, gave a catalytic response in 1 out of 2 samples of minced liver, but not with liver slices. The average Q_{O_2} for the minced liver was 3.6; for corresponding liver slices it was 5.9. The response of minced liver to fumarate and citrate was generally the same, but liver slices invariably showed greater increases in respiration with fumarate. Table V gives data from one of the liver experiments.

TABLE V

The effect of fumarate and citrate on the respiration of rat liver slices and mince.

Supplements	Oxygen uptake in U_{O_2}			
	Slices		Mince	
	1st hr.	2nd hr.	1st hr.	2nd hr.
0—Control	5.2	9.2	3.6	4.3
0.001 M fumarate	8.8	15.5	6.5	7.3
0.001 M citrate	7.6	11.7	4.5	5.4

Tumor tissue (Flexner-Jobling rat carcinoma) was less sensitive than most of the normal tissues studied. Tumor mince in Ringer-phosphate solution (containing glucose) had a Q_{O_2} of only 2.3. This material did not respond to fumarate, citrate, malate, succinate, *kochsaft*, cytochrome-c, or various combinations. Tumor slices, however, had a higher rate of respiration, $Q_{O_2} = 7.3$. They did not respond to the "C_4 acids" alone, but in the presence of *kochsaft*, a 30 percent increase in

respiration was observed when 0.001 M fumarate, malate, or citrate were added. Boyland and Boyland have reported that fumarate increases the respiration of the Jensen rat sarcoma, and of the Crocker rat sarcoma 180 (14). An interesting peculiarity of the tumor slices was the persistence of the respiration. Even after 4 hours the slices were respiring at their initial rate (Fig. 4).

Fig. 4. The effect of fumarate and allied substances on the respiration of slices and of mince of Flexner-Jobling rat carcinoma. 0.001 M succinate, fumarate, malate, and citrate; 0.2 cc. *kochsaft*. Broken line indicates minced tissue.

Summarizing, there appears to be abundant evidence that the "four carbon acids" are active in a wide variety of tissues. It may be that they function in all tissues, for the failure to demonstrate increased respiration on the addition of more acid obviously does not mean that the acid already present is inactive. On the other hand, an extreme opposite view probably should be stated, namely, that the increases in oxygen uptake observed *in vitro* may be merely artifacts of the laboratory, without any significance in the living animal, in as much as the demonstration of "catalysis" invariably depends on a certain amount of tissue damage. Corroborative evidence, however, suggests that the truth lies somewhat nearer the former view than the latter.

The various forms of evidence leading to the revised concept of hydrogen transport are discussed in detail in Szent-Györgyi's book "Studies on biological oxidation and some of its catalysts". Briefly the evidence is as follows: Tissues contain a mechanism for the very rapid reduction of oxalacetic acid, the end product being malic acid (8, 16). The kinetics of the reaction are such that all of the hydrogen mobilized in respiration could be carried by this means. Having shown that the succinate-fumarate system reacts with cytochrome, whereas the malate-oxalacetate system acts with the substrate, it remained to link the two systems (25). Green (26) showed that the two systems could be linked by means of suitable electroactive hydrogen transmitters, dyes, the end products being succinate and oxalacetic acid. The transfer of hydrogen from malate to fumarate could be shown with dyes and also with cytochrome (27). Finally Banga demonstrated the transfer of hydrogen from donator to cytochrome by means of the double system: oxalacetate-malate and fumarate-succinate (28). The natural intermediate appeared to be a flavoprotein (29). The "old yellow enzyme" from yeast has a potential midway between that of the two "C_4 systems" (30), and Laki (31) has shown that the yellow enzyme can be reduced by malico-dehydrogenase and that it can transfer hydrogen to the succino-dehydrogenase system. However, in view of the many flavoproteins recently isolated (32) it is most unlikely that the "old yellow enzyme" of yeast is the active flavin in the mammalian cell. More likely prospects would be either the adenine-alloxazine proteid (M.B.—red. coenzyme) of Haas (33) which reacts very rapidly with methylene blue though not with molecular oxygen, or the heart flavoprotein of Straub (34) which is believed to be identical with the coenzyme factor (35).

One of the weaknesses of Szent Györgyi's scheme of hydrogen transport is that it does not explain the catalysis due to citric acid. Using suspensions of pigeon breast muscle in phosphate buffer, Krebs and Johnson (2) showed that the extra oxygen consumed in the presence of citrate was much greater than could have been used up by this citrate itself. We have repeatedly confirmed this observation using Ringer-phosphate medium (see above) and furthermore, have observed increases in respiration due to citrate in a wide variety of tissues although mol for mol citrate was usually less effective than fumarate. With pigeon muscle in phosphate Krebs and Johnson observed that citrate catalysis was much more pronounced in the presence of carbohydrates; hexose phosphate, glycogen, or α-glycero phosphate. In our experiments with kidney slices, glucose itself made possible the demonstration of a catalytic effect. The assumption, therefore, is that citrate catalyzes carbohydrate oxidation. Krebs and Johnson added citrate to respiring tissues and observed that it rapidly disappeared (2). In the presence of arsenite, which inhibits the oxidation of α-keto acids, α-keto glutaric acid accumulated. In the presence of malonate, succinic acid accumulated. In each case the "derivative" accumulated in amounts comparable to the citric acid which has disappeared. It was therefore postulated that citric acid was converted first to α-keto glutarate, then to succinate. The first step had already been accomplished *in vitro* by Martius and Knoop (36, 37).

With the demonstration that citrate acts catalytically, and the further demonstration that respiring tissue destroys citrate, it followed that a mechanism must be present for the regeneration of citrate. This was shown by incubating oxalacetic acid with muscle mince anaerobically. Citric acid was formed in large amounts. On this basis the citric acid "cycle" was formulated (Fig. 2). Szent-Györgyi and co-workers had shown that oxalacetate could be reduced directly to form malate and eventually succinate (8, 16); Krebs and Johnson showed that oxalacetate could be oxidized, presumably after condensation with a triose, to form citrate and then by further "oxidation" (dehydrogenation plus decarboxylation) form succinate. Experiments with malonate seemed to indicate that both pathways are possible (2). Oxalacetate, plus malonate, incubated with tissue aerobically yielded more succinate than when oxalacetate alone was used, the assumption being that the malonate, by checking succinodehydrogenase, prevented further oxidation of the succinate previously formed by oxidation. Under anaerobic conditions a different phenomenon was observed. Actually more succinate was formed from oxalacetate than under aerobic conditions, presumably by reduction over malate and fumarate. In the presence of malonate, however, anaerobic succinate production was reduced, since

the inhibited succino-dehydrase lay in the direct path of succinate formation.

Krebs' citric acid cycle has been questioned by Breusch (38) who was unable to duplicate either an enhancement of citrate catalysis in the presence of glycerophosphate, or the formation of citrate from oxalacetate on incubation with muscle or liver. The first observation had been used by Krebs and Johnson (2) to link citrate catalysis with carbohydrate metabolism, the second, to indicate the mechanism of citrate regeneration. Breusch (38) contends that the citrate formed in Krebs' experiments arose by an unphysiological route, namely the formation of a condensation product *in vitro* when oxalacetic acid was neutralized, which condensation product was subsequently decarboxylated on incubation to form citric acid. In the absence of this pre-formed condensate, Breusch observed no citric acid formation from oxalacetic acid. On the contrary, malic acid was formed. Breusch further points out that citrate catalysis is usually somewhat delayed, as if the citrate had first to be converted to some other compound before it became active. Part of the discrepancy may have been due to differences in pH and in medium (plain phosphate by Krebs and Johnson, Ringer-phosphate by Breusch). It is noteworthy, however, that the catalytic activity of citric acid, iso-citric, *cis*aconitic, and α-keto glutaric acid were confirmed by Breusch, who was unsuccessful in establishing either of two possible explanations of his own for their activity. Thus, the Krebs cycle, though open to question in certain details has nevertheless served to focus attention on a new group of catalytic substances, the "C_6 acids."

Recent experiments of our own throw some doubt upon the specificity of malonate as an inhibitor for succino-dehydrogenase, since the effect of citrate on normal muscle was more effectively nullified by malonate than the effect of succinate itself. A few typical experiments will serve to illustrate the point. Using 0.001 M fumarate, succinate, and citrate, a marked stimulation of respiration was observed (Fig. 5). 0.001 M malonate inhibited respiration 31 percent; 0.01 M inhibited it 71 percent. When 0.001 M malonate was added along with 0.001 M fumarate, a 17 percent decrease in respiration was observed over the respiration of fumarate alone (U_{O_2} at 2 hours = 19.0 as compared to 23.0). The effect of 0.001 M succinate was reduced 16 percent by malonate (U_{O_2} at 2 hours = 16.0 as compared to 19.0). The effect of citrate, however, was reduced 42 percent (U_{O_2} at 2 hours with citrate = 24.0; citrate + malonate = 14.0). With excess malonate (0.01 M) approximately 70 percent inhibition was observed in the presence of 0.001 M
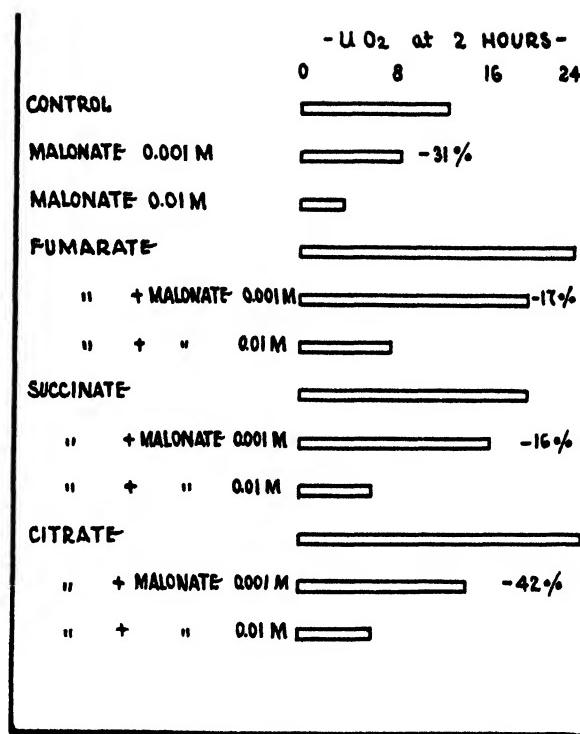
Fig. 5. The inhibiting effect of malonate on the respiration of pigeon breast muscle. 0.001 M fumarate, succinate and citrate; malonate in concentrations indicated.

succinate, fumarate, or citrate. The abnormal sensitivity of citrate was revealed even more clearly when higher concentrations of the salts were used. 0.005 M malonate inhibited respiration 60 percent; (U_{O_2} at 2 hours = 5.7 as compared to 14.2 for the control). Low concentrations, 0.001 M, of fumarate, succinate, or citrate stimulated respiration as before. When equal amounts of fumarate and malonate, 0.005 M, were added to muscle, respiration was practically the same as that observed in the presence of 0.001 M fumarate (U_{O_2} at 2 hours = 22.5 and 23.3). When equal amounts of succinate and malonate were added, respiration was equal to control respiration, but not equal to the oxygen consumption observed in the presence of low succinate only. Control, 14.2; 0.005 M succinate + malonate, 15.0; 0.001 M succinate, 23.1; 0.005 M malonate, 5.7 U_{O_2} for two hours. Increasing the amount of succinate to 0.01 M in the presence of 0.005 M malonate increased the U_{O_2} to 19.7.

In the presence of citrate, malonate inhibition was much stronger than in the presence of succinate. When equal amounts of citrate and malonate were added, respiration was not even restored to normal. Control, 14.2; 0.005 M citrate + malonate, 9.3; low citrate 0.001 M, 16.6 U_{O_2} for

2 hours. Increasing the amount of citrate to 0.01 M in the presence of 0.005 M malonate failed to increase the respiration; in fact, higher concentration of citrate actually depressed respiration somewhat (U_{O_2} for 2 hours = 7.8). It thus appears that malonate is not as specific in its action as hitherto supposed, a conclusion also reached by Weil-Malherbe (39).

A consideration of the curves of oxygen uptake indicated that the inhibiting effect of malonate was delayed for at least 20 minutes in the presence of succinate or fumarate, but in the presence of citrate the inhibiting effect of malonate manifested itself at once. This may have been due to purely physical phenomena, such as the relative rates of penetration of the substances into the cell. Some such explanation may also be involved in the frequent observations that citrate is not quite as effective in stimulating respiration as the other salts of the series. A study of the action of malonate on purified enzyme systems, as well as a study of accumulated end products, would appear highly desirable.

An interesting application of "C_4" and "C_6" catalysis has been the use of these substances in demonstrating an action of insulin *in vitro*. Krebs and Eggleston (18) proceeded from the premise that many factors are necessary in respiration, which may fail *in vitro* from a lack of any number of them. Hence they attempted to supply as many factors as possible. When enough other materials were added, insulin became the limiting factor, and insulin added to the mixture increased respiration.

A suspension of pigeon breast muscle in M/10 phosphate, pH 6.8, was used in their demonstration. It was supplemented with citrate, and with deproteinized muscle juice (*kochsaft*) which contained substrates, coenzymes, and inorganic ions. The juice stimulated respiration slightly, the citrate inhibited it. Juice and citrate appeared to counteract each other, since the addition of the two together gave a respiration approximately equal to that of the unsupplemented control. When insulin was added to such a "balanced" system, respiration was increased 90 percent. Insulin also increased respiration somewhat in the presence of citrate alone or of *kochsaft* alone. Both amorphous insulin and crystalline insulin hydrochloride were effective; but preparations containing zinc sometimes actually inhibited respiration. The experiments were continued for 4 hours.

Shorr and Barker (40) studied this effect on rabbit, cat, and dog muscle, and failed to observe any response to insulin. In pigeon muscle they observed a 20 percent increase in contrast to the 90 percent increase reported by Krebs and Eggleston. Banga, Ochoa, and Peters (41) observed stimulation due to insulin in certain brain preparations fortified with fumarate, adenylic acid, and pyruvate.

Our own experiments support the general conclusion that insulin may stimulate respiration *in vitro*. At first we attempted to repeat exactly the experimental conditions of Krebs and Eggleston (Table VI). Difficulties with their pipetting technique, however, forced us into weighing our tissue samples separately. In contrast to their results we observed a marked stimulation in respiration with citrate or *kochsaft* alone, and an even greater

TABLE VI

The effect of insulin and supplements on the respiration of pigeon breast muscle in M/10 phosphate buffer, pH 6.8.*

Supplements	Oxygen uptake				
	Total U_{O_2} values				4th hr. Q_{O_2}
	1st hr.	2nd hr.	3rd hr.	4th hr.	
0—Control	6.8	9.1	9.9	9.4	0
Citrate	13.5	19.8	22.1	23.5	1.4
Kochsaft	11.4	18.9	22.7	24.5	1.8
Citrate + *kochsaft*	14.1	22.9	27.1	29.1	2.0
Insulin + citrate + *kochsaft*	13.6	23.6	30.6	35.6	5.0
Fumarate + *kochsaft*	15.5	26.6	32.9	35.8	2.9
Insulin + fumarate + *kochsaft*	13.0	22.2	29.3	34.3	5.0

* 0.0134 M citrate and fumarate; 0.45 cc. *kochsaft*; 1.5 units amorphous insulin.

response when the two were added together (control U_{O_2} for 4 hours = 9.4, citrate plus *kochsaft* 29.1). Insulin plus citrate plus *kochsaft* increased the respiration 22 percent, the U_{O_2} being 35.6, which appeared to be about the maximum respiration for this tissue, since the same figure was reached by adding fumarate plus *kochsaft*, or fumarate plus *kochsaft* plus insulin. This effect was observed repeatedly.

Experimental conditions were therefore adopted which more nearly approached the physiological: Latapie mince, Ringer-phosphate pH 7.4, 0.001 M supplements of the "C_4 acids", 0.2 cc. *kochsaft* per 2 cc. of medium. Under these conditions *kochsaft*, fumarate, or 20 units of insulin[8] alone stimulated respiration about 20 per cent (Fig. 6). The respiration was "preserved" rather than accelerated. Any two of the supplements were more effective than one alone, in other words insulin increased respiration in the presence of fumarate or of *kochsaft*. Similarly, the three components were better than any two; insulin increased respiration in the presence of fumarate and *kochsaft*. However, insulin added only a 15 percent increment to a respiration which had already been stimulated 100 percent by fumarate and *kochsaft*. The effect was most pronounced during the last two hours of the experiment. Varying the amounts of insulin added between 2 and 40 units per flask, maximum stimulation was observed when 4 units were added to 200 mg. of tissue in 2 cc. of

[8] Unless otherwise mentioned the insulin was the Eli Lilly preparation known as Iletin.
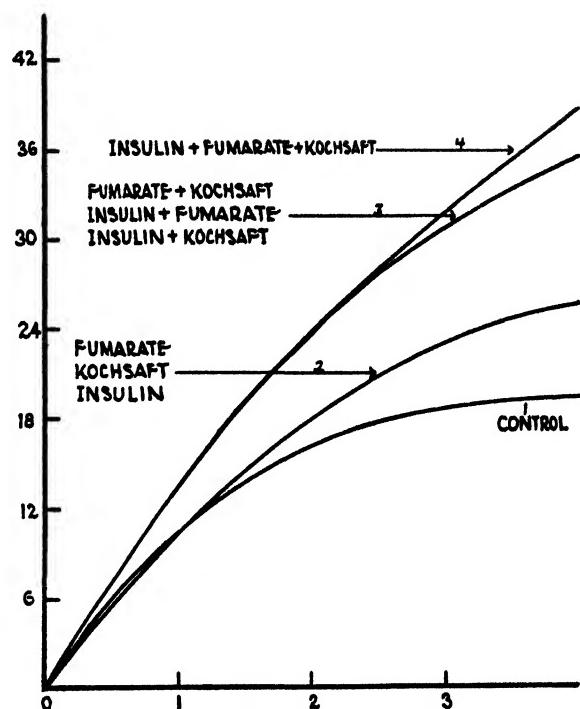
Fig. 6. The conserving action of insulin and fumarate on the respiration of pigeon breast muscle. 0.001 M fumarate; 0.2 cc. *kochsaft*; 20 units of insulin.

medium. Higher amounts decreased this effect, probably due to the preservative present in the insulin preparation. Essentially similar results were obtained when citrate (Table VII), succinate, or malate (Table VIII) was used in place

Table VII

The effect of insulin and of citrate on the respiration of pigeon breast muscle.*

Supplements		Oxygen uptake				
		Total U_{O_2} values				4th hr. Q_{O_2}
		1st hr.	2nd hr.	3rd hr.	4th hr.	
0—Control		12.6	20.7	23.3	26.2	2.9
Insulin, 4 units		15.4	26.0	33.6	39.5	5.9
" 8 units		14.9	24.7	32.5	38.4	5.9
" 20 units		12.9	23.2	29.7	34.4	4.7
" 40 units		11.8	20.2	26.6	32.0	5.4
Citrate		12.8	20.8	25.1	26.5	1.4
Kochsaft		12.8	23.3	29.2	30.6	0.4
Citrate + *kochsaft*		14.4	26.7	35.4	37.6	2.2
Insulin, 4 units + citrate		14.4	24.5	32.6	39.0	6.4
" + *kochsaft*		15.3	27.6	37.7	45.5	7.8
" + citrate + *kochsaft*		14.7	27.5	38.0	49.2	11.2

' 0.001 M citrate; 0.3 cc. *kochsaft*; 4 to 40 units of insulin as indicated.

TABLE VIII

The effect of insulin (Iletin and amorphous) and supplements on the respiration of pigeon breast muscle.*

Supplements	Oxygen uptake in U_{O_2}			
	1st hr.	2nd hr.	3rd hr.	4th hr.
0—Control	8.7	11.6	12.0	12.2
Insulin (Iletin)	11.8	13.8	14.1	14.3
" (amorphous)	10.3	13.0	13.3	13.5
Fumarate + *kochsaft*	13.9	23.5	30.3	32.8
Insulin (Iletin) + fumarate + *kochsaft*	13.9	24.2	33.4	35.2
" (amorphous) + " + "	14.6	24.0	31.5	37.2
Succinate + *kochsaft*	13.6	22.4	28.9	32.1
Insulin (Iletin) + succinate + *kochsaft*	13.3	22.6	30.3	35.2
" (amorphous) + " + "	13.4	23.6	31.1	36.0
Malate + *kochsaft*	12.8	22.0	28.6	31.3
Insulin (Iletin) + malate + *kochsaft*	15.8	27.3	36.7	39.7
" (amorphous) + " + "	12.9	22.3	30.0	35.7

* 0.001 M fumarate, succinate and malate; 0.2 cc. *kochsaft*; 4 units of insulin.

of the fumarate. Amorphous insulin[4] reacted similarly to Iletin.

We have repeated these insulin experiments on rabbit heart and skeletal muscle, and chicken breast muscle, in every case using Ringer-phosphate buffer. The effect was less striking with these lower respiring muscles but qualitatively the results were the same as those reported for pigeon breast muscle.

Summarizing, in experiments with insulin on surviving muscle, a slight stimulation of respiration was nearly always observed when the tissue was suitably supplemented. Sometimes the stimulation was quite appreciable. Insulin alone, however, was highly variable in its effect. Approximately one-third of the tissues showed some stimulation, one-third showed no effect, and one-third showed a slightly decreased respiration in the presence of insulin. A possible explanation of the variation may have been the variable amounts of insulin already present in the tissue. Hence, the effect of insulin was studied on the respiration of muscle from depancreatized pigeons. The general experimental procedure was the same as before: 150 to 200 mg. of Latapie mince, Ringer-phosphate buffer pH 7.4, 0.001 M "C₄ acids," 1 to 4 units of amorphous insulin or Iletin per 2 cc.

fluid. The experiments were performed one week to 10 days after pancreatectomy.[5]

Insulin alone markedly stimulated the respiration of muscle from depancreatized birds. In the first experiment the U_{O_2} at 4 hours was 19.0 for the control, and 38.0 in the presence of insulin, an increase of 100 per cent. As with normal muscle, fumarate plus *kochsaft* increased respiration markedly, and insulin in addition added another increment to the respiration. Since the rate of respiration was "preserved", the effect of insulin was most marked during the third and fourth hours of the experiment. In fact, during the latter half of this experiment insulin alone gave an increase of over 500 per cent as compared with the control (Fig. 7).

With a second depancreatized pigeon, insulin alone stimulated respiration 50 per cent, but insulin plus citrate plus *kochsaft* gave a 100 per cent increase over the effect of citrate plus *kochsaft* (Table IX). 0.001 M malonate markedly inhibited respiration stimulated by insulin. Again the effect was most marked in the third and fourth hours.

With a third depancreatized pigeon, 1 unit of amorphous insulin stimulated respiration 30 per cent; in the presence of *kochsaft* and "C₄", it stimulated respiration 50 per cent, in contrast to
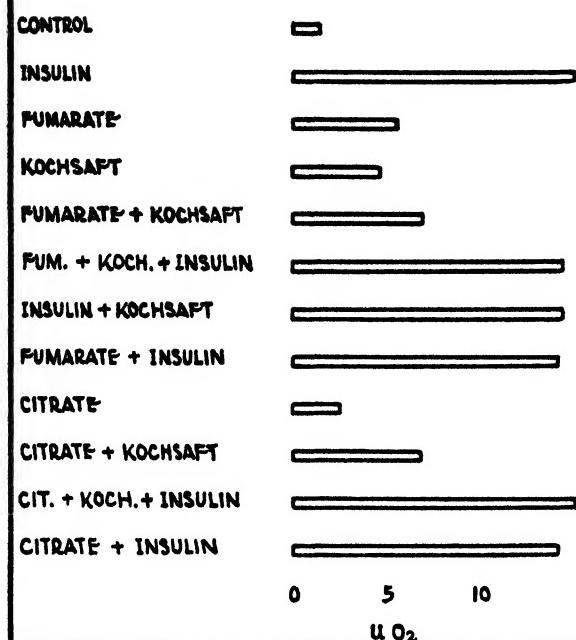
Fig. 7. The effect of insulin and supplements on the oxygen consumption of breast muscle from a depancreatinized pigeon, during the third and fourth hours of the experiment. 0.001 M fumarate and citrate; 0.2 cc. *kochsaft*; 4 units of insulin. Blood sugar before pancreatectomy was 184 mg. per cent; 9 days after, it was 210 mg. per cent.

the 20 per cent increase usually observed with normal muscle. "C_4", fumarate, citrate, malate, and succinate were interchangeable, and again the effect was most marked in the third and fourth hours.

The unexpected action of malonate in inhibiting citrate catalysis even more than succinate catalysis, has already been referred to. This effect is particularly striking in muscle fortified with insulin. For example, with our usual experimental conditions, the following 4 hour U_{O_2} values were observed: control, 28.2; Iletin (4 units), 26.7; Iletin + 0.005 M malonate, 5.1; 0.005 M citrate + *kochsaft*, 27.9; Iletin + citrate + *kochsaft*, 34.2; malonate + Iletin + citrate + *kochsaft*, 8.9 (Table X). The malonate apparently interfered with some system through which the insulin effect had been expressed. When fumarate was substituted for citrate, however, the inhibition due to malonate was usually negligible, as shown by the following 4th hour U_{O_2} values: control, 20.0; 0.005 M fumarate + *kochsaft*, 28.7; Iletin (4 units) + fumarate + *kochsaft*, 41.0; 0.005 M malonate + Iletin (4 units) + fumarate + *kochsaft*, 37.0 (Table XI).

The malonate, therefore, obviously had not inhibited the insulin directly. The results emphasize the extreme sensitivity of citrate to malonate, as well as the fact that a least a part of the "citric acid cycle" must be intact for insulin to exert an *in vitro* effect.

Table IX

The effect of insulin and supplements on the oxygen consumption of breast muscle from a depancreatized pigeon.*

Supplements	Oxygen uptake							
	Hourly Q_{O_2} values				Total U_{O_2} values			
	1st hr.	2nd hr.	3rd hr.	4th hr.	1st hr.	2nd hr.	3rd hr.	4th hr.
0—Control	14.7	8.9	3.5	1.0	14.7	23.6	27.1	28.1
Insulin, 1 unit	17.2	11.6	8.7	3.0	17.2	28.8	37.5	40.5
Amorphous insulin, 1 unit	17.8	12.5	7.2	2.7	17.8	30.3	37.5	40.2
Insulin, 4 units	19.2	12.8	6.0	3.4	19.2	32.0	38.0	41.4
Amorphous insulin, 4 units	17.3	10.4	6.9	4.4	17.3	27.7	34.6	39.0
Insulin, 4 units + malonate	4.8	1.7	0.3	0.2	4.8	6.5	6.9	7.1
Citrate + *kochsaft*	16.2	11.8	5.8	1.1	16.2	28.0	33.8	34.9
Insulin, 4 units + citrate + *kochsaft*	19.5	15.7	12.8	11.0	19.5	35.2	48.0	59.0
Insulin, 4 units + citrate + *kochsaft* + malonate	8.1	3.4	1.5	0.4	8.1	11.5	13.0	13.4
Fumarate + *kochsaft*	18.8	14.6	3.0	1.2	18.8	33.4	36.4	37.6
Insulin, 4 units + fumarate + *kochsaft*	17.8	15.2	11.0	10.0	17.8	33.0	46.0	56.0

* 0.001 M fumarate and citrate; 0.005 M malonate; 0.2 cc. *kochsaft*; 1 and 4 units of insulin and amorphous insulin as indicated. Blood sugar before pancreatectomy was 164 mg. per cent; 11 days after it was 216 mg. per cent.

TABLE X

The effect of malonate on the respiration of pigeon breast muscle stimulated by small amounts of insulin and citrate.*

Supplements	Oxygen uptake				
	Total U_{O_2} values				4th hr. Q_{O_2}
	1st hr.	2nd hr.	3rd hr.	4th hr.	
0—Control	12.1	20.7	26.6	28.2	1.6
Citrate + kochsaft	11.8	19.7	25.2	27.8	2.6
Insulin + citrate + kochsaft	12.0	20.6	28.0	34.2	6.2
Insulin + citrate + kochsaft + malonate	5.4	7.8	8.5	8.9	0.4
	(Malonate inhibition at 4 hrs. = 74 p.c.)				
Insulin	10.7	17.4	22.6	26.7	4.1
Insulin + malonate	3.5	4.4	4.7	5.1	0.4

* 0.005 M citrate and malonate; 0.2 cc. kochsaft; 4 units of insulin.

The inhibition of the insulin effect *in vitro*, whether directly or indirectly through the citrate cycle, raised the question whether malonate might not also hinder the action of insulin in the living animal. Accordingly, a 15 per cent solution of neutralized malonate was injected subcutaneously into rabbits, either before, or simultaneously with, a solution of insulin. The sugar content of the blood was then determined at intervals using the Shaffer-Hartman method. The exact dosages and the results of a preliminary experiment are given in Table XII. In rabbits No. 1 and 4, which received 4 units of insulin, the blood sugar fell rapidly and the animals went into convulsions. Rabbit No. 2 received the same amount of insulin, but in 2 cc. of molar malonate solution, and the blood sugar failed to decrease. Rabbits No. 3 and 5, receiving similar supplements, showed some decrease in blood sugar, though the decrease was much greater when insulin alone was given.

No. 6 received malonate 15 minutes before the insulin, and here again the blood sugar fell only slightly.

Table XIII gives the results of a more complete experiment. A study of this table shows that malonate retarded the action of insulin in lowering blood sugar for about an hour (rabbits No. 6 through 9, as compared with the first 5), but as time went on the malonate effect wore off and the blood sugar decreased. With the rabbits receiving malonate without added insulin (rabbits No. 10 through 14) there was a tendency for the blood sugar to rise slightly. No. 12 and 13, which received twice as much malonate as No. 10 and 11 showed a definite rise. No. 14 which received the same total amount of malonate as No. 12 and 13, but given at hourly intervals rather than in one dose at the beginning of the experiment, showed a marked temporary rise. The temporary effect of malonate in retarding the

TABLE XI

The effect of malonate on the respiration of pigeon breast muscle stimulated by small amounts of insulin and fumarate.*

Supplements	Oxygen uptake				
	Total U_{O_2} values				4th hr. Q_{O_2}
	1st hr.	2nd hr.	3rd hr.	4th hr.	
0—Control	13.6	18.5	19.5	20.0	0.5
Fumarate + kochsaft	13.4	21.9	28.4	28.7	0.3
Insulin + fumarate + kochsaft	14.7	24.3	33.9	41.0	7.1
Insulin + fumarate + kochsaft + malonate	15.0	23.6	31.4	37.0	5.6
	(Malonate inhibition at 4 hrs. = 10 p.c.)				

* 0.005 M fumarate and malonate; 0.2 cc. kochsaft; 4 units of insulin.

TABLE XII

The effect of insulin and malonate on the blood sugar of the rabbit.*

			Blood sugar in mg. p.c.		
			0 Hour	4 Hours	
Rabbit No. 1	4 units insulin		104 mg. p.c.	39 mg. p.c. (convulsion)	
" " 2	4 units insulin in 2 cc. of M/1 malonate		116 "	113 "	
" " 3	4 units insulin in 2 cc. of M/1 malonate		142 "	111 "	
			0 Hour	1 Hour	2 Hours
Rabbit No. 4	4 units insulin		101	80	56 (convulsion at 5 hours)
" " 5	4 units insulin in 2 cc. M/1 malonate		96	75	75
" " 6	10 cc. M/1 malonate, 15 minutes later given 4 units insulin		103	85	90

* Normal fed rabbits, 2 to 2.5 kg. in weight.

fall in blood sugar following insulin administration agrees with the results of Huszak (8) who observed that injection of malonate increases the excretion of acetone bodies for a period of about 2 hours.

These experiments are only preliminary. They are mentioned in the present form because they give some indication that reactions studied in the manometric vessel also proceed in the intact animal. Furthermore, they suggest another explanation for the great variability in insulin action, since insulin is only one of the factors involved in carbohydrate oxidation; others are fumarate, citrate, and the enzymes which activate them.

TABLE XIII

The effect of insulin and malonate on the blood sugar of the rabbit.*

			Blood sugar in mg. p.c. Hours			
			0	1	2	4½
Rabbit No. 1	4 units insulin		86	26 (convulsions)		
" " 2	" " "		93	46	47	54
" " 3	" " "		82	58	39 (convulsions)	
" " 4	" " "		98	52	45	39 (convulsions)
" " 5	" " "		83	51	42	35 "
" " 6	10 cc. M/1 malonate—15 min. later given 4 units insulin		96	83	55	37 (convulsions)
" " 7	" " " "		100	104	61	64
" " 8	20 cc. M/1 malonate—15 min. later given 4 units insulin		83	70	66	36 (convulsions)
" " 9	" " " "		77	85	(died—no convulsion)	
" " 10	10 cc. M/1 malonate		93	108	97	96
" " 11	" " "		96	114	114	115
" " 12	20 cc. " "		106	110	121	123
" " 13	" " "		84	109	102	135
" " 14	10 cc. M/1 malonate at beginning, repeated at end of 1st hour		110	99	212	107

* Starved (24 hours) rabbits, 2 - 2.5 kg. in weight.

There is ample evidence that fumarate, malate, oxalacetate, citrate, and succinate function catalytically in the biological oxidations of isolated tissues. Insulin is somehow connected with this catalytic cycle. The cycle catalyses the oxidation of carbohydrate. Malonate, under appropriate conditions, inhibits the cycle. There is some evidence that these *in vitro* experiments are representative of reactions in the intact mammalian organism.

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DISCUSSION

Dr. Burk: There is one idea which I think was not brought out that lends much support to Szent-Györgyi's original mechanism. The idea has been somewhat expressed already by Elliot, namely, that citrate should be regarded as an incidental, perhaps unnecessary, source of the malate and fumarate, and that in this sense the Krebs cycle is incidental to Szent-Györgyi's cycle which may be regarded as a fundamental cycle. It seems to me that this view is supported throughout by the new data just presented, especially in regard to the new point that malonate inhibits citrate action more easily and more quickly than it does fumarate or succinate action. This would be necessarily true, if citrate were a source of fumarate and succinate, under the conditions when the latter are limiting· One would get a greater and quicker inhibition in the case of citrate, because the succinate-fumarate system would, for a while at least, be more limiting than if the succinate and/or fumarate were added directly and adequately. The same logic holds with regard to the insulin results, all indications being that both citrate and insulin enter somewhat ·incidentally (even if not wholly) into respiration and its catalysis. The malonate acts solely and directly at the fumarate-succinate position when it does act.

Dr. Stare: Our data suggest that citrate is not a necessary part of the 4 carbon dicarboxylic acid system, but that it probably serves as a source of catalyst, presumably of succinate. We have not stressed this point, but may I refer again to data we have just given which indicate that citrate is not an essential component of the so-called Szent-Györgyi "cycle". The inhibition caused by 0.005 M malonate can be completely overcome by addition of succinate or fumarate in suitable concentration, but not by citrate. If succinate and fumarate functioned in a "cycle" of which citrate was an integral component, they could not overcome malonate inhibition, because malonate effectively blocks out citrate. This then indicates that citrate is not a necessary link in succinate-fumarate catalysis, but the fact that it acts catalytically in the absence of malonate suggests that it may serve as a source of catalyst.

With regard to insulin, it may not enter directly into fumarate catalysis, but our data suggest that it certainly has something to do with cellular respiration. Its action is emphasized in the experiments with breast muscle from depancreatized pigeons (see Table X).

Our data do not permit us to determine whether malonate acts solely at the fumarate-succinate position, although that is one possibility. It appears equally possible, however, that malonate is a more general inhibitor, affecting other systems, as for example, citrate.

Dr. Shorr: As Stare pointed out, Barker and I looked for the Krebs insulin effect in minced muscle of a number of mammals, including the dog, cat, and rabbit, and failed to find it. It is even absent in the breast muscle of the chicken. In view of Stare's observation that the insulin effect with pigeon breast mince is increased by using a Ringer-phosphate solution instead of water-phosphate, it may be profitable for us to repeat our experiments with this variation in the medium. I wonder whether Stare has any data as to the concentration of chlorides in the system as employed by Krebs, in comparison with the medium used by him. One would expect that the boiled muscle juice would contain all the chlorides present in the tissue used to prepare it. Furthermore, the mince used in the experiment would contribute its salts readily to the medium. The question of tonicity *per se* can hardly be expected to be significant when preparations are used whose cellular structure is completely destroyed. In surviving tissue slices proper tonicity becomes important. Slices of cardiac tissue show an enormous stimulation of respiration in boiled muscle juice uncorrected for tonicity, but this is soon followed by a sharp fall.

The fact that we obtained the insulin effect only with the pigeon, of all the species used, made us wonder whether there might be some peculiarities in the character of the metabolic processes in this bird. We were surprised to find that the respiratory quotient of the mince was in the neighborhood of unity no matter what the nutritional state. Even after a four day fast, which is extremely rigorous for a pigeon whose metabolism is so high, such respiratory quotients were also obtained. Blood sugar levels, which are ordinarily higher in pigeons than in mammals, persisted at these levels after fasts of three and four days. We then compared the respiratory quotient of the pigeon mince with that of muscle slices prepared simultaneously. Such slices, if carefully made, contain a high percentage of relatively intact muscle bundles and maintain their respiration very well. The respiratory quotients of the slices were at the low levels to be expected after a fast, while the quotients of the mince prepared from the same muscle were

around 1.0. Mincing, therefore, immediately changes the nature of the foodstuffs being oxidized, bringing about an almost exclusive oxidation of carbohydrates, to judge from the respiratory quotient. Under such conditions, the effect of insulin on the respiratory rate must be considered as a maintenance of a type of metabolism which is also going on in its absence, rather than a change in the character of the metabolites oxidized. A demonstration of a transition from fat to carbohydrate metabolism would be more convincing.

I am glad that Stare has shown that the insulin effect is far from being a maintenance of the original level of respiration. If the curves are plotted to show the respiration for each period studied, the marked falling off with time with the pigeon as well as all the mammalian tissues which we have studied, becomes apparent. The insulin effect is generally observed only at low levels of respiration, and is most striking towards the end of the experiment, when the total oxygen consumption in both the insulin vessels and the controls is small.

Dr. Stare: We have no data on the relative chloride contents of our system and that used by Krebs. There are, no doubt, chlorides present in *kochsaft*, but the amount of *kochsaft* used by us was comparatively small. We tried to add just enough to increase the sensitivity of the tissue to the "C₄" acids without increasing the oxygen uptake materially in the absence of "C₄" acids. This amounted to 0.1 to 0.2 cc. per 2 cc. of medium. Krebs used nearly 5 times that amount. Of course, if one adds more *kochsaft*, the respiration is increased enormously.

In all of our experiments with muscle we have used a "Latapie mince," and I should like to mention that mincing with the Latapie does not begin to completely destroy the cells. Microscopic examination of the mince shows a large number of intact cells.

In comparing our results with those of others, we should like to emphasize the "time factor" as well as the use of the Ringer-phosphate type of medium. We feel it is very important to work at the utmost speed from the time the tissue is excised until the cocks of the manometric vessels are closed. With all of our experiments this has been less than 25 minutes.

As for the peculiarity of the pigeon, pigeons seem to get along all right without a pancreas, at least for two weeks. They do not lose weight, nor does the blood sugar go up as much as one might expect (from 180 to 200-210 mg. p.c.). They also seem to stand a lot of insulin. We have given pigeons as much as 150 units of insulin within 24 hours; the blood sugar was reduced to about half of the normal level.

Dr. Shorr: Some of the experimental variations employed by Barker and myself in attempting to repeat Krebs' work were fasting and pancreatic diabetes in the dog. Under these conditions, as well as with skeletal muscle from cats, rabbits, and the chicken, both fed and fasted, we failed to observe any stimulation of respiration with insulin. I should like to ask another question about the blood sugar changes noticed after the injections of malonate in the rabbit. Were the rabbits fasted, and if so, for how long?

Dr. Stare: Twenty-four hours.

Dr. Shorr: What would be the respiratory quotient of the rabbit after a twenty-four hour fast?

Dr. Barker: Fasting a rabbit for that short length of time probably would not have any effect on the R.Q.

Dr. Shorr: I brought up the point by way of suggestion that you might choose experimental conditions for your malonate experiments which might be more decisive in determining their significance for carbohydrate metabolism. If malonate were given to animals whose carbohydrate metabolism was reduced to a minimum, as in pancreatic diabetes, it might be possible to ascertain whether the effects were widespread or localized, for example, in the liver.

Dr. Barker: Continuing the discussion of the effect of malonate on the whole animal, I should like to consider the significance of this phenomenon in relation to the effect of insulin on the oxygen consumption of the excised tissue. The preponderant *in vivo* effect of insulin is on storage rather than on oxidation, and any effect on oxidation of carbohydrate probably extends over a longer period of time. The effect of insulin *in vitro,* in the case of pigeon muscle, is to give a pick-up in oxygen. I wonder if you have been able to reconcile these two aspects. The situation in the whole organism may be confused by the glycogenolytic effect of malonate, which your data show. The result of this process would be to obscure the usual hypoglycemic effect of insulin. Your data, I believe, show a considerable increase in blood sugar after the injection of malonate alone, and variable effect after insulin plus malonate. This is just what one would expect if the delicately adjusted mechanisms for blood sugar regulation were disturbed by agents affecting both sides of the equilibrium.

Shorr and I always found marked stimulations with citrate, often amounting to 25 to 50 per cent of the initial value. The effect was duplicated in all of the animals we tried, including mammals, and yet the mammalian tissue did not give us any insulin stimulation, whereas the pigeon tissue did. Actually, in the case of the mammals, insulin decreased the oxygen consumption of the mince-

phosphate-*kochsaft*-citrate system. In view of Krebs' experiments we thought that this depression might be due to the zinc content of insulin. The Lilly amorphous preparation we used contained less than 0.025 per cent zinc, and we have successfully ruled that out by experiments with zinc alone. Our conclusion is that the depression may well be an effect of the insulin.

Dr. Stare: The interpretation of our results with malonate *in vivo* must, of course, depend upon what insulin actually does in the living animal. If the decrease in blood sugar in insulinized rabbits is entirely due to a withdrawal of sugar into storage, this phenomenon would necessarily concern an aspect of insulin activity different from its effect in increasing the oxygen consumption of tissues *in vitro.* But whatever the interpretation, the observations are as reported.

Mr. MacLeod: There has been some discussion in this paper about the effect of a lack of and an excess of certain ions on tissue metabolism. Then, too, we are made aware of differences obtained in the use of phosphate alone and phosphate plus NaCl. In other words, in many cases the medium employed has a determining effect on the amount or type of metabolism which we may expect from certain tissues. It is obvious, I think, even under the best of *in vitro* conditions where various forms of Ringer media are used, that one is left with a doubt as to the validity of certain results. I have wondered why the workers in the field do not take the best step towards the use of a physiological medium and employ serum. True, there are certain technical difficulties in the use of serum which require careful work, but they are not so difficult nor time consuming that they render the use of serum impossible.

Serum increases the respiration of exudate leucocytes about 70 per cent relative to the respiration of the same cells in Ringer-phosphate. The same is true of bone marrow and several other tissues.

Since isolated cells and tissue slices behave differently, depending on the fluid medium employed, it is still to be shown that their response to certain substances may not depend also on the nature of the medium.

Dr. Baumann: Certainly the respiration of isolated cells depends on the nature of the medium in which they are placed. But in the study of the "C$_4$" acids, there is some question whether a "perfect physiological medium" would be desirable. The experimental conditions which we try to attain are those in which all factors necessary for respiration are present except the C$_4$ acids. This means that the medium must be sufficiently unphysiological to permit these substances to diffuse away, or to be destroyed, so that the tissue will respond when normal concentrations are restored.

An analogy might be made with the study of vitamins, in which the ideal ration is one containing all necessary factors except the one being studied, so that the animal can respond when that factor is added.

Dr. Barker: I was not quite clear whether you said you always needed glucose present in order to get your citrate effects.

Dr. Stare: With kidney, yes; with the other tissues, no. Occasionally when we have done experiments with muscle without glucose, we did not observe the usual effect with C_4 acids. So as a matter of precaution we put it in. With kidney we found it necessary.

Dr. Barker: Of course we have used a much higher citrate content than you have; however, with slices of heart muscle we have been able to get marked citrate stimulation of respiration in Ringer-phosphate.

Dr. Stare: We have never tried a heart slice.

Dr. Burk: What is the relative concentration of insulin added compared with serum protein which exists in serum? I wonder whether there is any correlation between mere protein concentration, of insulin, or serum protein, and the insulin effect. If you were to add other non-specific proteins, what would happen?

Dr. Stare: I don't know. In our insulin experiments we have used amounts varying from one unit to forty units per vessel (total volume = 2 cc.)

Dr. Warren: There are two points about which I wish to comment. One is about the use of serum. It is interesting that you can use neutralized serum in tissue work and obtain the same stimulating effect you do with untreated serum. This can be done in an ordinary Warburg apparatus, and in the case of glycolysis too there is about a 70 per cent increase in the neutralized serum compared with Ringer's solution. I would like to inquire whether you have any information about the action of these C_4 substances on glycolysis? When you increase respiration do you decrease aerobic glycolysis accordingly?

Dr. Stare: I don't know and we have not any information of our own.

Dr. Müller: There has been a good deal of discussion about damaged cells and I should like to know what we mean by a damaged cell.

Dr. Warren: Whereas we obviously cannot answer this question fully, it is pertinent to realize, as MacLeod has mentioned, that in serum the rate of respiration (of bone marrow at least) does not fall off markedly as time goes on (as in Stare's experiments) but remained absolutely constant for at least five hours. Part of the damage to the cells, then, must be due to the absence in salt solution of something that is present in serum. Three groups of workers have tried to identify this substance (or substances) but each used a different tissue and each obtained a different result. Fat soluble substances, diffusible substances and proteins have accordingly been proposed; our own experiments on bone marrow are in progress and we do not have an answer yet.

Dr. Shorr: What is the significance of the R.Q. in the presence of succinate? When succinate produces a marked stimulation of respiration, you are inclined to interpret the low R.Q. as due to surplus dehydrogenation exceeding the oxidative capacities of the system. It would seem to me that such an interpretation of the low R.Q. in the presence of a substrate would only be justified if there were no change in oxygen consumption. Whereas, in this instance, oxygen consumption is greatly increased. There is no direct evidence that the rate of dehydrogenation is actually exceeding the oxygen-transporting capacity of the cell.

Dr. Baumann: Perhaps more direct evidence would be an analysis of the system for incompletely oxidized fragments of succinate Such fragments (fumarate, malate) are said to accumulate when fairly large amounts of succinate are added to respiring tissue. The extra oxygen consumption (in addition to respiration) could then be regarded as combining with hydrogen from the succinate to form water. If this view be correct, a lowering of the R.Q. must necessarily follow, and that, in fact, is what one observes. What we really tried to indicate was that the agents transporting hydrogen from succinate to oxygen are extremely active, capable apparently of transporting amounts of hydrogen from succinate equivalent to all of the hydrogen mobilized in normal respiration. This would furnish some justification for including the succinate-fumarate transformation as one step in the various "cycles."

Dr. Ochoa: Stare and Baumann's extensive data (from experiments performed on suspensions of tissue containing preformed substrate) on the activation of the respiration of various tissues by C_4 and C_6 acids do not inform us as to the nature of the carbohydrate (H_2 donor), the oxidation of which is being catalytically stimulated by those acids. Banga and Szent-Györgyi (1) had already given evidence to show that a donator, in muscle respiration, is triosephosphate. Annau (2) obtained evidence that pyruvate can be another such donator in muscle. Our own experiments on brain dispersions, in which most of the preformed substrate and coenzymes are removed by dialysis, supply new as well as complementary information for this discussion. These experiments clearly show that the C_4 dicarboxylic acids (succinate, fumarate, malate) are also concerned with the oxidation of pyruvate. When brain dispersions (pigeon, rabbit) in 0.9 p.c. KCl

are dialysed for a short time (against 0.4 p.c. KCl) their power to oxidise added pyruvate is almost completely lost, but can be fully restored by addition of inorganic phosphate + a C_4 dicarboxylic acid + "adenine nucleotide" (3). In order to restore the oxidation of pyruvate to the level before dialysis, the presence of a C_4 dicarboxylic acid is essential, as indeed is that of any other of the substances mentioned above. The effect of the C_4 dicarboxylic acids is catalytic, their addition increases the net O_2 uptake of pyruvate from over 100 to 200 p.c. The following typical figures to illustrate this point are taken from a paper recently submitted for publication (4).

converted by transamination (6) into glutamic acid. Krebs and Cohen (7) have recently presented evidence to show that the system α-ketoglutaric acid $\rightleftharpoons$ glutamic acid can act catalytically as a hydrogen carrier.[*]

Dr. Stare: The experiments reported by Ochoa add more weight to the importance of fumarate in tissue respiration. Since the oxidation-reduction system succinate $\rightleftharpoons$ fumarate has a potential of E'_0 0.00 volt at pH 7.0, 38° C., it is thermodynamically possible that it may act as a mediator for the electron transfer in biological oxidations of systems with a lower oxidation-reduction potential, such as the oxidation of pyruvate where

Enzyme = pigeon brain dispersion (dialysed 2.5 hours) + phosphate + adenylic acid + Mg^{++}. (μl oxygen uptake in 20 min. at 38° C.)

	No addition	0.005 M fumarate	0.005 M citrate
Enzyme	36	81	40
Enzyme + pyruvate (0.009 M)	146	322	186
Net O_2 uptake of pyruvate	110	241	146

The effect of citrate. The above table raises another point. It may be seen that the catalytic effect of citrate on the oxidation of pyruvate by the brain dispersions is much smaller than that of fumarate, and also than that of other C_4 dicarboxylic acids (4); in some experiments it has been *nil*, and it does not replace the C_4 dicarboxylic acid, *i.e.*, with citrate, the original level of respiration before dialysis is not restored. In view of this it has already been pointed out (3) that oxidation of pyruvate in brain cannot be catalysed by a citric acid cycle. If such were the case, the effect of citrate should have had to be identical to that of C_4 dicarboxylic acids. Also, if a citric acid cycle were operative, any other intermediate of the cycle should have had to be as active as a C_4 acid; this did not prove to be the case with α-ketoglutarate (4).

Now Stare and Baumann also find in most cases, and with various tissues, smaller effects of citrate mol for mol. Their evidence thus reinforces our own (*cf.* also Breusch, 5) and extends it to the case of muscle tissue oxidising its own preformed (or added) carbohydrate. The obvious inference, that the citric acid cycle is unlikely to be operating in a main pathway of oxidative breakdown, is not drawn by the authors. Tentative explanations for the effect of citric acid (when present) might, however, be (*a*) supplying of C_4 acids by oxidative breakdown, citric → *cis*-aconitic → *iso*-citric → α-ketoglutaric → succinic acid, or (*b*) α-ketoglutaric acid may be partially

the E'_0 value is about −0.180 volt at pH 7.0, 38° C. This apparently happens in the experiment reported by Ochoa, and future research will probably show other systems where the electron transfer goes *via* fumarate.

The poor effect of citrate in the experiment reported by Ochoa may have been due to the absence of triphosphopyridine nucleotide, as Adler and Euler have recently shown that the dehydrogenation of *iso*-citric acid requires this coenzyme.

Dr. Peters: Ochoa's remarks cover most of the points in relation to the C_4 and C_6 acid cycle raised by work in this laboratory. It may be added that ever since the start of work upon the effect of vitamin B_1 *in vitro*, we have always been aware that the oxidation of succinate appeared to be entirely distinct from that of lactate-pyruvate (1). This gave no justification for the belief that succinate was part of the oxidation system of the latter. This view was especially reinforced by the observation that with a brain *brei* oxidising succinate, the extra oxygen uptake produced by addition of pyruvate was an additive quantity (2). Now that the C_4 acids have been proven to be catalytic and therefore to have some relation

* References: (1) Banga and Szent-Györgyi, Hoppe-Seyler Z. *252*, 275 (1938). (2) Annau and Erdös, Hoppe-Seyler Z. *257*, 111, (1939). (3) Banga, Ochoa and Peters, Nature, *144*, 74 (1939). (4) Banga, Ochoa and Peters, Biochem. J. (in press). (5) Breusch, Hoppe-Seyler Z. *250*, 262 (1937). (6) Braunstein and Kritzmann, Enzymologia *2*, 129 (1937). (7) Krebs and Cohen, Nature, *144* (1939).

to the oxidase system, this point needs further discussion because in theory there is a possible path for conversion of succinate to pyruvate *via* oxaloacetic acid; it is quite clear that any such conversion is much too slow to be a main path for the oxidation of pyruvate. It is a fact that avitaminous pigeon brain tissue shaken with succinate or fumarate (3) shows evidence, after a time, of the formation of traces of pyruvate (as judged by the nitroprusside reaction). This must be formed *via* the path fumarate → malate → oxaloacetate. In the presence of sufficient pyruvate, however, this reaction is unlikely to occur to any extent. The question has not been worked out for small concentrations of pyruvate under these conditions, but Green (4) found that the activity of muscle

malate dehydrogenase was inhibited 76 p.c. by 0.06 M pyruvate. It is therefore likely that in the presence of pyruvate, conversion of fumarate to pyruvate *via* oxaloacetate tends to be inhibited. There seems to be here a beautiful example of biological adaptation; fumarate (or succinate) can be slowly formed either *via* pyruvate (5) or possibly α-ketoglutarate, because the C_4 system is needed for proper functioning of the total pyruvate oxidase system. Excess fumarate so formed can escape back to pyruvate again.**

** References: (1) Gavrilescu, Meiklejohn, Passmore and Peters, Proc. Royal Soc. London, B, *110*, 431 (1932). (2) McGowan and Peters, Biochem. J. *31*, 1637 (1937). (3) Unpublished experiments, Peters and Wakelin. (4) Green, Biochem. J. *30*, 2095 (1936). (5) Weil-Malherbe, Biochem. J., *31*, 2202 (1937).

AN ANALYSIS OF THE PYRUVIC ACID OXIDATION SYSTEM

Fritz Lipmann

The experiments on pyruvic acid oxidation to be described were undertaken as a continuation of studies on the Pasteur effect (1). To find a satisfactory solution of the problem of the Pasteur reaction it became necessary to learn more about the catalytic reactions involved in the later stages of carbohydrate oxidation. Pyruvic acid, the oxidant in glycolysis, is reduced under anaerobic conditions to lactic acid; under aerobic conditions it is oxidized ultimately to carbon dioxide and water. Dissociation between aerobic and anaerobic carbohydrate breakdown might take place, therefore, at the pyruvic acid stage.

Peters and his collaborators discovered the connection between thiamin (vitamin B_1) and the oxidative breakdown of pyruvic acid. To acquire a more definite knowledge of the role played by thiamin was one of the main purposes of this work. Before reporting on an analysis of the enzymatic reactions, however, attention should be called to some aspects of the chemistry of this key substance. The structure of thiamin was revealed by the work of R. R. Williams and his collaborators, who found that the molecule is composed of two parts: a thiazole and a pyrimidine ring. The pyrimidine is connected through a methylene bridge with the thiazole nitrogen, which thereby becomes quaternary. A quaternary pyridine had previously been found to be the reacting part of the prosthetic group of the enzyme dehydrogenating hexose -6- phosphate. [Warburg in collaboration with Karrer (2), *et seq.*] This same pyridine, nicotinic acid amide, was found in cozymase, the hydrogen transporting agent in alcoholic fermentation and in a great number of dehydrogenation reactions (Warburg, v. Euler). The catalysis of all these dehydrogenations consists fundamentally in an alternating hydrogenation and dehydrogenation of the quaternary pyridine:
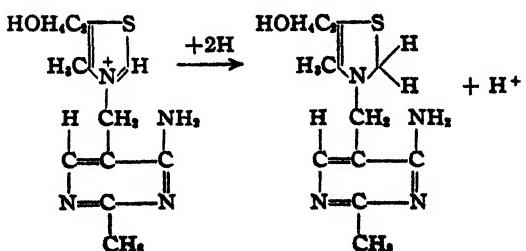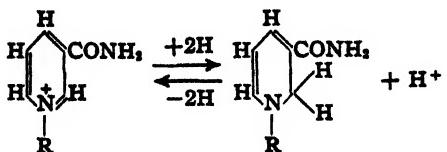
The well known chemical similarity between thiazoles and pyridines, and the similarity in their biochemical function as codehydrase components, induced me to see whether the ability to add hydrogen at the double bond in the neighborhood of the quaternary N was likewise shown by thiamin. In collaboration with G. Perlmann (3), it was shown that by treating thiamin and other quaternary thiazoles with sodium hydrosulfite or platinum-H_2, hydrogen addition actually takes place in the same manner as with the pyridines (2). But whereas the pyridine in the codehydrases of Warburg and von Euler (nicotinic acid amide) gives a stable product of dehydrogenation, due to the stabilizing effect of the acid amide group in *meta* position to the quaternary nitrogen, the hydrogenated thiazoles, including thiamin, appeared to be unstable, with the exception of benzothiazole. In benzothiazole the hydrogenated product is stabilized by the benzene ring attached to the thiazole. It can be assumed that with the quaternary thiazole acting as the active part of the pyruvic acid dehydrogenase a similar stabilization of the hydrogenated compound is brought about by its attachment to the enzyme protein. Up to now, however, it has not been finally proved that the catalytic action of thiamin pyrophosphate as part of the dehydrogenation system is due to alternating hydrogenation and dehydrogenation. As our work went on, it was thought best to postpone a more detailed study of the thiamin function until the function of the many other components participating in this surprisingly complicated dehydrogenation were better understood.

Shortly after the hydrogenation of thiamin had been obtained (3, 4), Lohmann discovered that thiamin pyrophosphate was the prosthetic group of yeast carboxylase (5). Influenced by Lohman's work, it was believed almost universally—and can be found in many textbooks—that the sole function of thiamin was to catalyze the decarboxylation of pyruvic acid to acetaldehyde and CO_2. It became evident, however, from the subsequent work of Krebs (6), Lipmann (7), Weil-Malherbe (8), and Peters (9), that pyruvic acid in animal tissues and in many bacteria is broken down only in the presence of a suitable hydrogen acceptor, and that the first products of its breakdown are acetic acid and CO_2; when dismutation occurs pyruvic acid itself is the hydrogen acceptor. The breakdown of pyruvic acid by simple decarboxylation into acetaldehyde and CO_2 is thus not as ubiquitous as had been thought.

In all cases where a cell uses pyruvic acid as such, and not its breakdown product, aldehyde,

as the oxidant in anaerobic carbohydrate metabolism (lactic, not alcoholic, fermentation), carboxylase is not to be found in appreciable amounts. It seems quite probable that pyruvic acid breakdown goes through the stage of simple decarboxylation exclusively in most, but not all, organisms belonging to the plant kingdom. In the animal kingdom, and in bacteria, pyruvic acid is broken down by dehydrogenation, and, in numerous bacteria, by the so-called hydroclastic reaction: "hydrolysis" to acetic acid and formic acid. It is my impression that a fundamental likeness exists between the mechanisms of the dehydrogenation and of the hydroclastic reactions, both complicated reactions, which phylogenetically might have been developed from the relatively simpler process of decarboxylation [see Barron (10)].

Characteristics of the Bacterial Dehydrogenation System

Working with animal tissues I found the pyruvic acid dehydrogenation system quite unstable, so I tried to find elsewhere a similar but more stabilized system. Quite early, Barron (10, 11) had become aware of the importance of a-keto acid dehydrogenation. He worked with resting gonococci and analysed a very interesting case of purely oxidative breakdown of pyruvic acid to acetic acid and CO_2. According to his experience, however, the gonococcus system was also quite unstable. Fortunately I became aware of a paper by Davis (12) describing certain lactic acid bacteria, *Bact. acidificans longissimum,* that contain a powerful pyruvic acid dehydrogenation system, stable enough to withstand drying with acetone. This system appeared to be well suited to withstand the rough treatment necessary to separate the components, so that it could be analysed as the fermentation system of the yeast cell had been analysed previously. The analysis was greatly simplified by two characteristics of the bacteria: oxidation only to acetic acid and CO_2, and absence of iron-containing oxidation catalysts (13).

The experiments were carried out with preparations dried either with acetone (14), or *in vacuo* over phosphorus pentoxide. From vacuum-dried young bacteria very active enzyme extracts were obtained by shaking with secondary phosphate. The enzyme solutions thus obtained could be centrifuged for 15 minutes at 15,000 r.p.m. without appreciable loss of activity. When kept dry in the refrigerator these vacuum-dried preparations stayed active for over a year; acetone bacteria were somewhat less stable, their activity dropping after one or two months. The pH optimum for our system is situated between 6.5 and 6.

It was found that at least five components are required for the catalysis of pyruvic acid dehydrogenation with oxygen as hydrogen acceptor: (1) thiamin pyrophosphate, (2) flavin-adenine dinucleotide, (3) Mn^{++} or Mg^{++} or Co^{++}, (4) protein(s), (5) inorganic prosphate. Components (1) and (3) are the same as in the carboxylase system of Lohmann, whereas components (2), (4) and (5) are specific for dehydrogenation. As will appear later, the participation of phosphate is the most important difference between dehydrogenation and simple decarboxylation. The separation of the above components and their significance in the reaction will now be described in detail.

Thiamin component.

Acetone bacteria were the most suitable material for studying this component separately. On washing the bacteria two or three times with 0.1 molar phosphate of pH 8 only traces of activity remained. With most preparations the other components were not removed by this treatment. The procedure is practically the same as that used for the extraction of cocarboxylase from yeast [Auhagen (15), Lohmann and Schuster (5)]. The measurements shown in Table I were made with the residue after three washings; 10γ of flavin-adenine and $100\ \gamma\ Mg^{++}$ as sulfate were added to each cup, besides sodium pyruvate (0.2 molar final concentration). The experiment was designed primarily to determine, if possible, the dissociation constant of the thiamin part of the enzyme.

To calculate from such data the dissociation constant of the thiamin-pyrophosphate-protein compound two well-justified assumptions must be made:—first, that the catalytic effect is entirely due to the combined product; second, that the concentration of protein-bound thiamin-pyrophosphate is negligible compared to its total concentration in solution. The velocity of oxidation, v, measured as O_2 consumption, is then proportional to the concentration of combined compound. The velocity at saturation ($V_{max.}$) is proportional to the concentration of total enzyme protein present, and $V_{max.} - v$ therefore is proportional to the concentration of uncombined protein. The concentration of added thiamin-pyrophosphate is c.

Then according to mass action law:

$$\frac{c(V_{max.} - v)}{v} = \qquad (1)$$

$$\frac{(\text{th.p.p.})\ (\text{uncombined protein})}{(\text{th.p.p.-protein})} = K$$

TABLE I.

Dissociation of the thiamin pyrophosphate-protein: the thiamin-part of pyruvic acid oxidase.

thiamin pyrophosphate $\gamma/cc.$	$-O_2$ mm.^{3}/15 min.			
c	v	$1/c$	$1/v$	c/v
(0.04)	4			
0.29	28	3.5	3.7×10^{-2}	1.0×10^{-2}
0.54	42.5	1.9	2.3	1.3
1.0	62	1.0	1.6	1.7
2.0	95.5	0.5	1.05	2.1
4.0	118	0.25	0.85	3.4
10.0	129	0.1	0.79	7.8
30.0	132	0.033	0.77	22.8

This equation was used successfully by Warburg and Christian (16) to calculate the dissociation constant of the flavoprotein d-amino acid oxidase.

The assumption that saturation of the protein was approximately reached at our highest concentration (30 γ th.p.p./cc.) yielded a value for $V_{max.}$ of 132. It appeared, however, that K in equation 1 was fairly constant for the lower values of c, but fell greatly for the higher values. Burk suggested trying a graphical method of calculation introduced by himself and Lineweaver (17). One of the great advantages of this method is that no experimental value of $V_{max.}$ is needed, for it can be calculated from the observed lower values of v and c. Being derived from the Michaelis-Menten (18) equation (which is derived from equation 1), the method was used for calculations of the enzyme-substrate dissociation type. It may be pointed out that in the present and similar cases the coenzyme is the equivalent of a substrate.

Lineweaver and Burk transform the Michaelis-Menten equation:

$$v = \frac{V_{max.} \times c}{K + c} \qquad (2)$$

into a linear form by taking the reciprocals of both sides:

$$\frac{1}{v} = \frac{1}{c} \cdot \frac{K}{V_{max.}} + \frac{1}{V_{max.}} \qquad (3)$$

Multiplying this by c yields another linear form:

$$\frac{c}{v} = c \cdot \frac{1}{V_{max.}} + \frac{K}{V_{max.}} \qquad (4)$$

FIGURE 1.

FIGURE 2.

The molecular weight of thiamin-pyrophosphate is 480 according to Lohmann. Therefore:

$$K = 1.3 \text{ mg./liter} = 1.3/480,000 = 2.7 \times 10^{-6} \text{ mol/liter}$$

In Table II the dissociation constant of our thiamin-enzyme complex is compared with that of other dissociating enzymes measured in Warburg's laboratory.

Of the three representatives of the vitamin B group, the greatest affinity (*i.e.,* the lowest dissociation) is found with the flavin-enzymes; the thiamin-enzyme is intermediate, and the nicotinic acid-enzyme is the lowest. The dissociation constant between the prosthetic group and its protein is of great biological significance, since it indicates roughly the concentration of the respective group needed in the cell to develop full enzymatic activity. Thus, in rat liver cells, for example, the concentration of thiamin pyrophosphate is only 10γ per gram dry weight [Westenbrink (21)] against 1000γ of cozymase [Elvehjem (22)], *i.e.* 1 to 100, corresponding to a ratio of the dissociation constants of 1 to 33 (see Table II.)

Flavin component.

Quite early during this work (14), it appeared that the oxygen consumption of washed acetone bacteria, when saturated with thiamin pyrophosphate, could be greatly enhanced by addition of purified flavin (-adenine) preparations obtained from ox heart (Table III). At the same time, simple riboflavin was inactive. Recently, identification of the catalytically active flavin compound became possible (20) by the use of a method developed by Warburg and Christian (16) to remove the flavin part from the protein part of *d*-amino acid oxidase.

Enzyme solutions obtained from bacteria in the manner described above, were precipitated repeatedly with ammonium sulfate of 50 p.c. saturation at pH 3 in the cold. This procedure yielded a protein fraction practically free of flavin, thiamin and metal compounds. This is illustrated by the experiment shown in Table IV. Here the precipitate was finally dissolved in phosphate to give a pH of about 6, and sufficient amounts of Mg were added.

For the moment only the aerobic experiment A will be considered. Oxygen consumption appears

From either equation 3 or 4, K and V_{max} can be derived by plotting $1/v$ against $1/c$, or c/v against c, respectively. In the first case $1/V_{max}$ is the ordinate intercept and $1/K$ is the slope; in the second case $1/V_{max}$ is the slope and K/V_{max} is the ordinate intercept.

Both methods were used in our case and gave very satisfactory straight lines (Fig. 1 and 2). The dissociation constants calculated from both curves were nearly the same, 1.3 and 1.34 (γ/cc.), respectively. The computed values for V_{max} were correspondingly 150 and 153. It appears that our initial failure to obtain a constant with equation 1 was due to the use of a much too low value for V_{max}, namely 132 instead of 150. This shows the great advantage of Lineweaver and Burk's method, which implies no assumption as to the true V_{max} value, which is frequently very difficult to obtain for one reason or another.

TABLE II

	dissociation constant mol/liter	
d-amino acid oxidase (flavin-adenine-protein)	2.5×10^{-7}	(16)
pyruvic acid dehydrogenase (thiamin pyrophosphate-protein linkage)	2.7×10^{-6}	
alcohol dehydrogenase (diphosphopyridine-protein)	9×10^{-5}	(19)

TABLE III.

Activation of pyruvic acid oxidation by thiamin pyrophosphate, flavin preparation from heart and extracts of animal organs (14).

Acetone bacteria, washed twice, with phosphate of pH 8, finally suspended in 0.1 M phosphate of pH 6. Total volume per cup 1.65 cc. containing 0.03 M-sodium pyruvate and *ca.* 33 mg. acetone preparation.

Thiamin pyrophosphate	Other additions	O_2 consumption/30 min.
		mm.[a]
—	—	3.5
—	50γ thiamin	4
4	—	74
12	—	96
12	0.30 mg. riboflavin	95
12	flavin-"flavin phosphate" from heart	168
—	brain extract = 4 gm. fresh brain	145
—	kidney extract = 3.5 gm. fresh kidney	180
—	dto., but without pyruvate	12

when Warburg's flavin-adenine dinucleotide is added, together with thiamin pyrophosphate. The preparation used was a pure compound kindly supplied to me by Prof. O. Warburg. The flavin component is evidently identical with that of *d*-amino acid oxidase [Warburg and Christian (16)], xanthine oxidase [Ball (23)], and dia-

phorase [Corran, Green and Straub (24)]. This "diaphorase" from heart seems to be equivalent to the methylene blue reducing yellow enzyme obtained from yeast by Haas (25). These flavoproteins apparently connect, with additional links, the pyridine codehydrogenases with the iron containing catalysts. In our system, which does not contain iron catalysts (13), the flavoprotein reacts directly with oxygen, as do the flavoproteins *d*-amino acid oxidase, the old yellow enzyme, and xanthine oxidase. It is inserted as a link between thiamin and oxygen. It is tempting to assume that in the animal cell thiamin is linked up through a flavoprotein like diaphorase in the same manner as the pyridines. The necessity of a flavin intermediate emphasizes the similarity between thiamin and pyridine-dehydrogenases.

Dismutation.

Before considering the anaerobic experiment some remarks must be made about pyruvic acid dismutation in our lactic acid bacteria. One of the great advantages of *Bact. acidificans longissimum* for the study of pyruvic acid dehydrogenation was that preparations of it can easily be obtained showing great activity *aerobically*, but almost no activity under *anaerobic* conditions (see Table IV). This indicates rather strongly that the oxidative reaction cannot be divided into an anaerobic and an aerobic part, thereby precluding decarboxylation or dismutation reactions as prephases in pyruvic acid oxidation.

It was observed, however, that anaerobic breakdown can be developed by the addition of free riboflavin. The reaction thus obtained is the dismutation:

$$CH_3 \cdot CO \cdot COOH(H_2O) + CH_3 \cdot CO \cdot COOH =$$
$$CH_3COOH + CO_2 + CH_3CHOH \, COOH$$
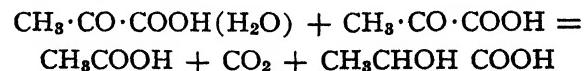
TABLE IV.

Effect of thiamin pyrophosphate, flavin-adenine and riboflavin on enzyme solution after precipitation with 50 p.c. ammonium sulfate at pH 3 and 2° C. (2).

	1	2	3	4
dissolved precipitate cc.	1.0	1.0	1.0	1.0
0.1 M MgCl cc.	0.1	0.1	0.1	0.1
thiamin pyrophosphate, γ	15	—	15	15
flavin-adenine, γ	20	20	—	20
riboflavin, γ	150	150	150	—
A. *aerobic* — mm.[3] O_2/25 min.	238	24	10	248
B. *anaerobic* — mm.[3] CO_2/90 min.	232	9	2.5	31

Other dyes, so long as they are not reduced entirely like methylene blue, can replace flavin, but have a much smaller effect. The function of the dye must be that of a hydrogen carrier combining two enzymes otherwise not reacting with each other (Borsook, Green). The view that two different enzymes participate in the reaction was strengthened by specific inhibition experiments. Dismutation is strongly inhibited by oxalate and by tartronate, which inhibit lactic acid oxidation but not pyruvic acid oxidation. Similar experiments with animal tissues had been carried out by Quastel (26).

Coming now to the anaerobic experiment, experiment B in Table IV shows that dismutation as well as oxidation is catalysed by the combined action of thiamin pyrophosphate and flavin-adenine, which indicates the participation of pyruvic acid dehydrogenase in the dismutation reaction. Dismutation was measured manometrically by measuring CO_2 formation. It seems worthwhile to emphasize that here also, where the hydrogen acceptor is not oxygen, but the dye (flavin or another) acting as intermediate, the thiamin part of the dehydrogenase acts only by intermediation of the flavin-adenine part of the enzyme system. According to these observations pyruvic acid dismutation is to be formulated as follows:

$$\text{pyruvic acid} \xrightarrow{+2H} \text{lactic acid}$$

lactic dehydrogenase

$\boxed{2H}$ ...... flavin

flavin-adenine compound

---- pyruvic dehydrogenase

thiamin pyrophosphate

$$\text{pyruvic acid (H}_2\text{O)} \xrightarrow{-2H} \text{acetic acid} + CO_2$$
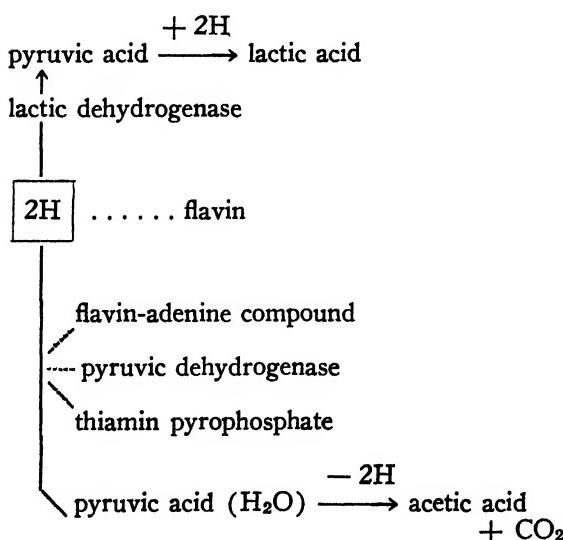
The lactic acid system of our bacteria was not investigated more closely. It appears that lactic acid oxidation is not abolished by the various treatments carried out to remove the prosthetic groups of the pyruvic system. Nor did addition of cozymase enhance lactic acid oxidation or pyruvic dismutation.

Hydrogen peroxide formation.

Since in our enzyme system the hydrogenated flavin-adenine-protein reacts directly with oxygen, hydrogen peroxide must be formed. Hydrogen peroxide was not found, however, for as is well known, pyruvic acid (here always in excess) reacts very rapidly without any catalyst with hydrogen peroxide (27). Acetic acid and CO_2 are the products of this reaction. The intermediate formation of H_2O_2 could nevertheless be demonstrated when catalase was added in amounts large enough to compete with pyruvic acid for the H_2O_2. As shown by the experiment in Table V,

TABLE V.

Effect of catalase on pyruvic acid oxidation

catalase, cc.	0.2	0.05	0
—mm.^{3}O$_2$/30 min.	36.5	50	53.5

oxygen consumption is lowered greatly with enough catalase present to decompose the intermediately formed H_2O_2. By reason of this result it must be concluded that half of the pyruvic acid is oxidized enzymatically, whereby H_2O_2 is formed (reaction 1 and 1a with omission of the protein parts), which oxidizes without catalyst the other half (reaction 2), both oxidation processes yielding acetic acid and CO_2:

thiamin pp.

1) $CH_3COCOOH$ ..... flavin-adenine $=$
$CH_3COOH + CO_2 + H_2$-flavin-adenine

1a) H_2-flavin-adenine $+ O_2 =$
flavin-adenine $+ H_2O_2$

2) $CH_3COCOOH$ (H$_2$O) $+ H_2O_2 =$
$CH_3COOH + CO_2 + 2H_2O$
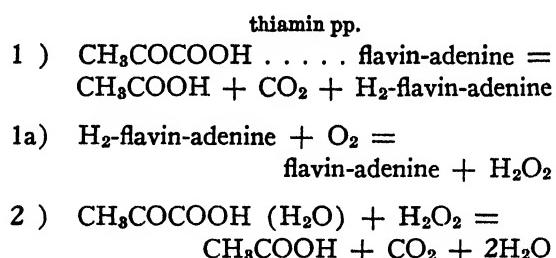
Controls have shown that reaction 2 is by far fast enough to account for the immediate disappearance of H_2O_2, and that an extra catalyst for this reaction is unnecessary.

Metal component.

This component is common for carboxylase and pyruvic dehydrogenase. Lohmann and Schuster (5) had found that either Mg^{++} or Mn^{++} must be present to complete the carboxylase system. Cobalt^{++} acts with both enzyme systems in the same manner. With dried bacteria, after washing at pH 8 or 6 or 4.7, and on addition of Mn or Mg, only insignificant effects could be observed. But by precipitation of the enzyme solution with ammonium sulfate at an acid reaction, as described above, the metal component was removed. Table VI shows the effect of the ions, and in particular the superiority of Mn^{++}, likewise observed with carboxylase. The chlorides were used for the experiment,

TABLE VI.

Effect of Mn++, Mg++ and Co++ on pyruvic acid dehydrogenation.

added	mol/e	—mm.^{s}O$_2$/20 min.
Mn++	—	35
	2.5×10^{-3}	112
Mg++	—	29
	6.3×10^{-3}	104
Co++	—	25.5
	3×10^{-3}	77

Protein component.

Not much work has been done on the enzyme protein, because it is difficult to obtain amounts of bacteria sufficient to justify a serious attempt at protein purification. Up to now I have not succeeded in separating the protein into one fraction binding the flavin component and another fraction binding the thiamin pyrophosphate. Most of the activity is found in the fraction precipitated between 45 and 55 p.c. saturated ammonium sulfate. Such crude experiments do not preclude the possibility that the two prosthetic groups are bound to separate proteins, nor the possibility that both are bound to the same protein body. The experiments of Ball (23) on xanthine oxidase might be cited here as suggesting the participation of another diffusible component besides flavin-adenine, but both apparently bound to the same protein. Perhaps this other diffusible component is thiamin pyrophosphate itself.

Phosphate.

When the dried bacteria were washed with acetate buffer of pH 5 and afterwards suspended in acetate at pH 6, practically no oxygen uptake oc-

TABLE VII

Effect of phosphate and arsenate on phosphate-free acetone bacteria.

Phosphate 10^{-3} mol.	Arsenate 10^{-3} mol.	O$_2$ consumption per hour mm.^{s}
0.2	—	9.5
1.3	—	45
2.4	—	79
5.5	—	132
—	0.54	59
—	1.08	90.5
—	2.7	149
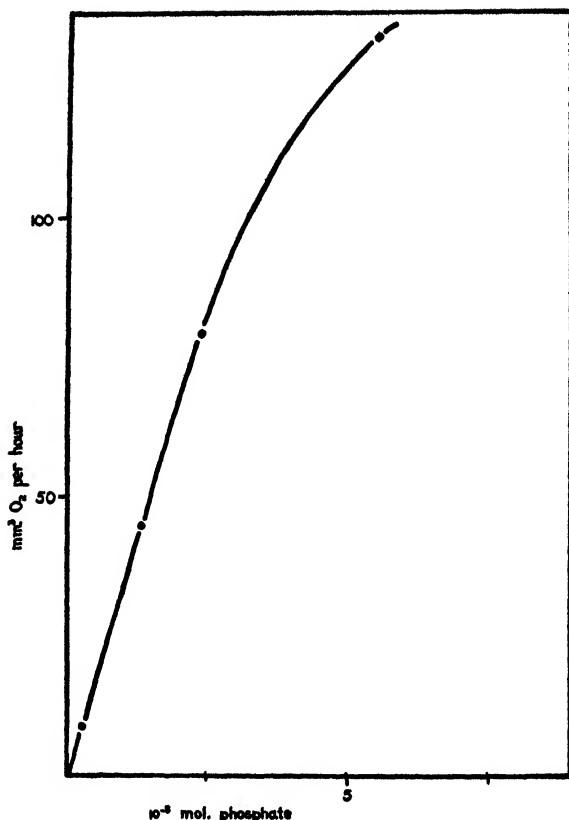
FIGURE 3.

curred with pyruvic acid alone, but full activation was found on addition of sufficient amounts of inorganic phosphate (14). (See Table VII, and Fig. 3.) Arsenate can replace phosphate entirely. Fig. 3 shows that up to a phosphate concentration of about 0.01 M the rate of oxygen consumption is proportional to the concentration. With about 0.015 M the system is saturated with phosphate. From these results the idea was formulated that phosphate is in some way intermediately bound during the process of dehydrogenation. From the work of Lohmann and Meyerhof the pyruvic acid-enol-phosphate $CH_2:COPO_3H_3 \cdot COOH$ is well known as an intermediate in fermentation. The P-O-C bond in this compound is quite an energy-rich bond. Though the intermediate formation of such a bond did not seem impossible for energetical reasons, the features of the reaction made it not very likely. For example, it was not possible to write the dehydrogenation of this compound without making further assumptions, and the formation of an energy-rich bond at the beginning would imply an initial starting energy that would have shown itself by the presence of an autocatalytic period at the beginning, which was not found. The intermediate formation of this com-

pound could be excluded almost with certainty when it was found that the phosphopyruvic acid of Lohmann and Meyerhof was inert in our dehydrogenation system (14).

The necessity for the presence of inorganic phosphate had been found with another dehydrogenation, that of phosphoglyceric aldehyde. Not only is the presence of phosphate necessary but, as Needham and Pillai (28) and Meyerhof and his collaborators (29) discovered, phosphorylation of adenylic acid is catalysed specifically by this dehydrogenation. Because of the conformity of the two reactions with respect to the need for phosphate, the coupling of dehydrogenation and phosphorylation with pyruvic acid dehydrogenation was tried (30).

TABLE VIII

	Initial value	0.125 M pyruvate	0.125 M pyruvate 0.03 M adenylic acid	0.03 M adenylic acid
Mg. inorganic P	0.59	0.53	0.31	0.59
Mg. easily hydrolysed P	0	0.06	0.28	0.01
—mm.^{3}O$_2$	—	490	474	58

The experiment shown in Table VIII was carried out with unwashed dried bacteria. Fluoride was added to slow the breakdown of phosphorylated compounds. It appears that in the presence, but not in the absence, of adenylic acid fairly large amounts of inorganic phosphate disappear and are quantitatively found in the easily hydrolysable phosphate fraction, indicating the formation of adenosine-triphosphate. It might be mentioned that with different preparations the accumulation of adenosine-polyphosphate was more or less marked. This was thought to be due to a more or less marked activity of adenylpyrophosphatase, as fluoride usually does not inhibit this enzyme sufficiently. This coupled reaction should be of major metabolic importance, as it represents the linking with phosphorylation of a reaction usually occurring only aerobically. It might, for example, account for the phosphorylation energy supplied to a muscle contracting under aerobic conditions. Such generalization seems justified since very recently Banga, Ochoa and Peters (31) have described the necessity of phosphate for pyruvic acid dehydrogenation in brain also. Furthermore, they have now found the coupling of

this dehydrogenation with adenylic acid phosphorylation with brain suspensions.

The observation of the coupling emphasized further the similarity between pyruvic acid and phosphoglyceric aldehyde dehydrogenation. There had been much speculation made over the mechanism of this coupling until very recently, when Negelein and Brömel (31) found the explanation in the case of the phosphoglyceric aldehyde. They describe the isolation of a labile diphosphoglyceric acid as the primary product of phosphoglyceric aldehyde dehydrogenation. According to their paper the real substrate for the dehydrogenation is not mono- but di-phosphoglyceric aldehyde, in that the di-phospho compound, in a reversible, apparently non-enzymatic, reaction is formed by addition of inorganic phosphate to the mono-phosphoglyceric aldehyde. The labile second phosphate group of the dehydrogenation product, di-phosphoglyceric acid, is transferred to adenylic acid, thus accounting for the coupling between the two reactions.

These observations in the Warburg laboratory, which clarify the need of inorganic phosphate and the catalysis of phosphorylation, strongly suggested a reformulation of the process of pyruvic acid dehydrogenation. The new formulation is illustrated by the following equations.

1) *Addition of phosphate:*
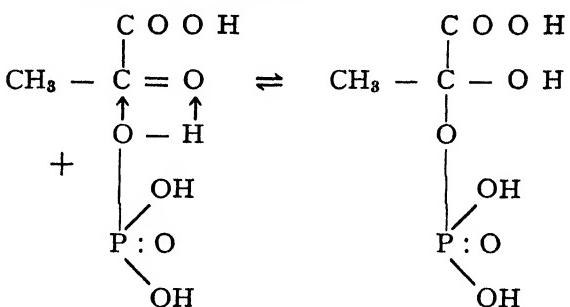
2) *Dehydrogenation:*

3) *Coupling:*

acetyl phosphate + adenylic acid →
    acetic acid + adenosine polyphosphate.
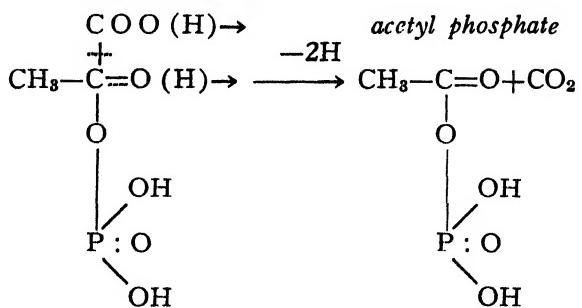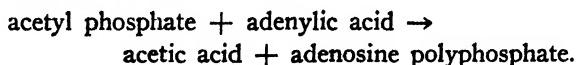
Equation 1. A reversible reaction between pyruvic acid and inorganic phosphate is assumed, in which a pyruvic acid phosphate is formed by addition of phosphoric acid at the carbonyl group. The addition product differs significantly from the phospho-enol-pyruvic acid of Lohmann and Meyerhof by the content of one more mol of water.

Equation 2. . The addition product is the real substrate for the dehydrogenating enzyme. By dehydrogenation one hydrogen belonging originally to phosphoric acid, and one belonging to the carboxyl group, are removed. In our formulation the phosphoric acid replaces the water whose addition was necessarily assumed earlier for dehydrogenation of carbonyl compounds (Wieland). This formulation accounts for the necessity of inorganic phosphate specifically required in the dehydrogenation of pyruvic acid by bacteria and in brain. Simple decarboxylation in yeast goes as well in the absence of inorganic phosphate as in its presence.

The products of dehydrogenation are acetyl-phosphate and CO_2. The formation of acetyl-phosphate corresponds with the formation of phosphoglyceryl phosphate in di-phosphoglyceric aldehyde dehydrogenation.

Equation 3. The energy-rich acetyl phosphate is able to phosphorylate adenylic acid. This accounts for the coupling between dehydrogenation and phosphorylation.

Anticipating great difficulties in the isolation of the supposed intermediate acetyl phosphate, an indirect method was chosen to test the theory, by testing the possibility of a phosphate transfer from acetyl phosphate to adenylic acid (equation 3). Acetyl phosphate had been prepared by Kämmerer and Carius (33) in 1864 from tri-silver phosphate and acetyl chloride. Primarily tri-acetyl phosphate, $(CH_3CO)_3PO_4$, is formed. The products of Kämmerer and Carius' reaction are dissolved in water, neutralized and some inorganic phosphate precipitated as barium phosphate. The bound phosphate, evidently a mixture of mono-, di- and tri-acetyl phosphate $[CH_3CO \cdot PO_4H_2, (CH_3CO)_2PO_4H$, etc.], remains in solution, and behaves much like creatine phosphate. It is surprisingly stable at neutral reaction, but easily broken down by strong acid even at room temperature.

Experiments like that reproduced in Table IX showed that 30 to 50 p.c. of the added acetyl phosphate were found in the pyrophosphate fraction, when excess of adenylic acid was present. Fluoride was added to slow down the decomposition of adenyl pyrophosphate and the experiments were done anaerobically to avoid input of oxidative energy.

The results of these experiments, in proving the

TABLE IX

Transfer of phosphate from acetyl phosphate to adenylic acid with bacterial preparations.

Fresh solution, containing 0.75 mg. acid-labile P in 0.5 cc., 46 mg. dry bacteria, total volume 1.25 cc., with 0.04 M in NaF and

mg. adenylic acid:	4	0.1	—
mg. P, inorg. + labile (acetyl.P):	1.12	1.39	1.37
mg. P, after 7' hydrolysis at 100° in normal HCl:	1.47	1.48	1.47
mg. pyrophosphate	0.35	0.09	0.10

occurrence of reaction 3, indicate strongly the correctness of our formulation given in the above equations. We showed above the occurrence of a coupling between pyruvic acid oxidation and phosphorylation, which would now find its explanation in the intermediation of a "phosphoryl acetate". On the other hand, the same substance, "acetyl phosphate", as an intermediate in pyruvic acid oxidation, and containing a highly reactive acetyl group, would not be entirely unexpected, considering the well known coupling between acetylation and pyruvic acid oxidation, as in the acetylation of amino acids (34) or of choline (35). The synonymous use of the terms phosphoryl acetate and acetyl phosphate is introduced only to point out the double activity of the compound and not by any means to encourage the use of the very unhandy term phosphoryl acetate.

In this survey, we have confined ourselves to a report of results obtained with the bacterial system. Only occasionally were some of these results related to reactions going on in animal cells. Nevertheless, these bacteria may be considered as a somewhat simplified model of the animal cell. The recent important results of Ochoa, Banga and Peters substantiate such a view. They also show that the system in brain, being intimately connected with the breakdown also subsequent to the acetic acid stage, is still more complicated, requiring, for example, succinate in addition to our components.

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DISCUSSION

Dr. Melnick: During the past year Stern and I have been doing some work on carboxylase in yeast. We have studied the relation between the prosthetic group and the protein carrier, and while the same relations may not hold for the case of the lactic acid bacteria studied by Lipmann, it may be of interest. When cocarboxylase is added to a fresh yeast extract, no additional decarboxylation

of pyruvic acid is noted, but after the extract has been ultracentrifuged at 512 r.p.s. the addition of cocarboxylase to the various layers produces an activation in the bottom layer only. This indicates that the protein carrier has been sedimented, and that in that fraction there is no longer present enough coenzyme to saturate the protein. This seems to place carboxylase in the same class as the pyridine enzymes, where one protein molecule catalyses the reaction between many coenzymes and substrate molecules.

We have also studied the reduction of thiamin, and have confirmed Lipmann's finding with respect to the reduction by hyposulfite and by platinum plus H_2. The reduced form of thiamin was found to be biologically inactive. On the other hand, when cocarboxylase was reduced with platinum-hydrogen the reduced form was found to be active both in the pigeon and in the yeast test system. This substantiates Lipmann's hypothesis that the thiamin nucleus undergoes reversible reduction in biological processes.

A third point concerns the postulate of semiquinone formation in the course of reduction. Lipmann noted that when thiamin was reduced with hyposulfite, a green-yellow color was developed, which suggested to him the possibility of semiquinone formation. We were unable to confirm this observation when the pH of the solution was maintained at about 7. However, if insufficient sodium bicarbonate was present during the reduction the pH decreased to about 3 and a green-yellow color appeared. But when Na-hyposulfite alone is brought to pH 3 the same color is observed.[1]

Dr. Lipmann: I can only say that we have found the green color in all cases where quaternary thiazoles are reduced, and we have found that the development of the color is about equivalent to the rate of reduction. We found with certain thiazoles that the reduction was much more rapid than with thiamin; and in such cases we also found that the color was correspondingly stronger; its development and disappearance were more rapid and it was visible even at low concentrations. In such cases the color can be seen in a reduction experiment with hyposulfite and bicarbonate in the Warburg vessel, and here certainly the pH must remain greater than 7. Further experiments should be done.

Dr. Melnick: Was the pH determined in your experiments with thiamin?

Dr. Lipmann: No, it was not determined; but calculating the amount of buffer present and the

[1] It is now recognized that the greenish colour appearing on reduction of thiamin with hyposulfite is due to formation of a greenish intermediate and not to acidification of hyposulfite. (See K. G. Stern and J. L. Melnick, J. Biol. Ch., 131, 597 (1939) footnote on page 608.)

amount of hyposulfite added, the acidity was certainly not as low as pH 3.

Dr. Melnick: Did you see any color with platinum and hydrogen?

Dr. Lipmann: I could never observe any. But when I reduced with zinc in acid solution I again got a very transient green color. Here the thiamin was broken down fairly rapidly and very soon a strong smell of hydrogen sulphide appeared. But the color can be seen if one works carefully and adds only small amounts of zinc. I made the experiment in an evacuated Thunberg tube with zinc in the side arm; on addition of zinc the color appeared for a few seconds.

Dr. Melnick: We tried reduction with zinc and HCl and we could not observe a color.

Dr. Lipmann: The visibility of the green color depends markedly on the concentration of thiamin. With concentrations below 1.0 per cent the color is very faint.

Dr. Stern: I might add to Melnick's remarks that we do not wish to throw any doubt on Lipmann's hypothesis that thiamin acts by virtue of reversible reduction. In fact, the biological activity of reduced thiamin pyrophosphate both *in vitro* and *in vivo* gives strong support to his hypothesis. But I think it should be made clear that at the present time no evidence exists for the electrochemical reversibility *in vitro* of the thiamin or any pyridine system. Attempts in various laboratories have failed to bring out electro-active reversibility in the cozymase system or in the thiamin system, either by potentiometric or by colorimetric methods, neither is there any proof for the intermediate semiquinone formation in these systems.

Concerning the interesting calculations which you and Burk performed with respect to the effect of thiamin pyrophosphate on the pyruvate oxidation by lactic acid bacteria, I wonder whether you are measuring the Michaelis constant of the formation of the compound between the coenzyme and the protein carrier, or that of the dehydrogenase-pyruvic acid complex.

Dr. Burk: The dissociation is with respect to coenzyme, added as catalyst, but without commitment as to whether the complex with which it unites involves merely the protein carrier or protein carrier and substrate together. Neither carrier nor substrate is involved as concentration functions in the expressions obtained. For the present, one cannot say just what total complex the thiamin pyrophosphate is combining with. The simplest assumption, if one is to be made, is that the complex would simply be the protein-thiamin. Substrate combination in addition might or might not be taking place, but other data must decide this point.

Dr. Stern: But if you measure the over-all

catalysis and if catalysis can ensue only when coenzyme is present to combine with the protein carrier, you may have been measuring both constants.

Dr. Burk: The perfect hyperbolic relation found to occur between rate and coenzyme catalyst concentration is decidedly against this unnecessarily complicated interpretation. This is an excellent example of where the beauty and simplicity of an observed mathematical relation (in analytical geometry) is very telling.

Dr. Cori: How was the concentration of the catalyst determined in that particular calculation?

Dr. Lipmann: It was the added thiamin pyrophosphate plus the very small amount present after three washings.

Dr. du Vigneaud: Have you ever tried to add to your bacterial preparations amino acids plus acetylphosphate, to see if any acetylation of the amino acids occurs?

Dr. Lipmann: I have not done the experiment. I think it would be most interesting to try.

Dr. Barker: In view of the importance of the phosphorylation and dephosphorylation steps, what do you consider takes up the phosphate instead of adenylic acid in your system?

Dr. Lipmann: It is possible that adenylic acid has to be present in catalytic amounts. It is also possible that an enzyme is present, which simply breaks down acetylphosphate. Nevertheless, in the presence of adenylic acid the phosphate is transferred to adenylic acid.

Dr. Barker: I noticed from your table that you obtained no better oxygen consumption in the presence of added adenylic acid. So there must be something besides added adenylic acid when the phosphate is taken up.

Dr. Lipmann: Not necessarily. But it could still be that adenylic acid must be present. Cori showed that adenylic acid catalyses phosphorylation of glycogen and dephosphorylation of glucose-1-phosphate without giving any sign of intermediate adenylic acid phosphorylation.

Dr. Barker: Can the phosphate simply be liberated without the necessity of any organic carrier?

Dr. Lipmann: That is possible. The only thing I can tell is that when I add adenylic acid, in addition to the other prosthetic groups, to the ammonium sulfate precipitate, which is probably my purest protein fraction, I cannot see any increase. Lipton mentioned, in connection with yeast, that adenylic acid sticks very much to the protein. The same might be the case with the bacterial proteins. Cori had to use electrodialysis to remove adenylic acid from his extracts.

Dr. Lipton: You would expect to find some pyrophosphate formation in the absence of any added adenylic acid, and apparently you do not.

Dr. Lipmann: The amounts are too small to measure. It is only a catalytic concentration of adenylic acid which might be present.

Dr. Lipton: If there is a small amount of adenylic acid present and you get alternative phosphorylation and dephosphorylation, then why, when you add a lot of adenylic acid, do you get a piling up of the pyrophosphate?

Dr. Lipmann: I agree with you, that in case you stop the breakdown of pyrophosphate, you ought to stop the reaction if only catalytic amounts of adenylic acid are present, and alternative phosphorylation and dephosphorylation have to occur. But that is not the case. Fluoride certainly inhibits dephosphorylation but has very little influence on pyruvic acid dehydrogenation in my preparations.

Dr. Lipton: Is it possible by some sort of analysis to demonstrate the formation of the acetyl-phosphate, for instance, with magnesium mixture?

Dr. Lipmann: It might be. I tried this earlier with magnesium mixture and did not find any acid-unstable phosphate by this procedure, but I have learned from my recent experiments that the breakdown of the intermediate is very rapid in acid solution, and I shall repeat the experiment under more favorable conditions. It might be possible, and it would be marvelous if I could isolate the intermediate.

Dr. Müller: Have you ruled out the possibility of the enol form of pyruvic acid entering into this reaction? All your illustrations are in the keto form and you state that the fermentation phosphopyruvic acid contains pyruvic acid in the enol form and gives no results. We know, however, that at pH 6 a considerable portion of pyruvate is in the enol form.

Dr. Lipmann: In my concept of the dehydrogenation pyruvic acid enters in the keto form. Only with the keto form can phosphate combine in an addition reaction. The addition must take place at the double bond between C and O in the keto group. For example, as it is well known, sulphite adds to keto groups in such a way. Now, when pyruvic acid is dehydrogenated it has to add either water or something else, because otherwise it cannot be dehydrogenated to acetic acid and CO_2. I have always written this water in the equations in parenthesis, because I have never been certain about real water addition. And now it appears that actually it is not water but phosphoric acid which is added, and I think for many reasons a much better picture now obtains.

Dr. Lipton: Coming back to the association of the coenzyme with the protein. In my yeast system I did not succeed in washing out any coenzyme at pH 6, where decarboxylation occurs. Secondly, I would like to comment again upon my experience where I get a very high degree of efficiency in the synthesis of thiamin pyrophosphate, but I can get just as much as corresponds to the maximum of activity and no more thiamin pyrophosphate will be synthesized.

Dr. Lipmann: I have thought about your experiment, and I think it is significant that you apparently got as much thiamin pyrophosphate formed as there is enzyme-protein present. This result is not easy to understand if a dissociation of the coenzyme-enzyme compound takes place.

ENZYMATIC BREAKDOWN AND SYNTHESIS OF CARBOHYDRATE

CARL F. CORI

This paper deals with the first stages of glycogen breakdown (and its synthesis) in animal tissues. The large glycogen molecule must first be broken down into smaller units before further reactions can occur. There exists a common initial pathway for fermentation and oxidation, the description of which will establish the necessary correlation with the main topic of this Symposium.

The matters to be considered are: (1) the enzymes which break down and synthesize glycogen, (2) the products formed by these enzymes, and (3) the points along the anaerobic pathway at which oxidations can occur.

Two enzymes are known to occur in animal tissues which are able to disintegrate glycogen, namely, diastase and phosphorylase. The physiological importance of these two enzymes in the breakdown of glycogen in such tissues as muscle, heart, brain and liver will first be considered. The fact that the end-products of glycogen breakdown in liver and muscle are glucose and lactic acid, respectively, has led to the assumption that from the very start two different enzyme systems are involved, and up to the present it has been a generally accepted idea that blood sugar is formed from liver glycogen by a diastatic enzyme.

Diastase. Animal diastases, when acting on glycogen *in vitro*, form dextrins of varying chain lengths, non-fermentable reducing substances, and maltose (1). Glucose formation is insignificant during incubation periods of 2 to 4 hours, while after 24 hours of incubation with strong enzyme solutions a considerable amount of the glycogen which has disappeared may be present as glucose. When glycogen is broken down in the intact liver, glucose is the chief product formed. Dextrins, non-fermentable reducing substances, and maltose do not accumulate to a measurable extent in the liver, nor can they be demonstrated in the blood stream. It might be said, of course, that the absence of intermediates is due to a special coordination of enzyme activity in the intact cell. Such a possibility cannot be denied, if one remembers that several intermediary products between glycogen and lactic acid accumulate in muscle extracts, but are not found in intact muscle.

Diastatic activity has frequently been demonstrated in liver and other organ extracts, but little attention has been paid to the fact that the blood of certain species contains a large amount of diastase. It was noted by Davenport (2) that extracts of perfused rabbit liver were very weak in diastatic activity, so that incubation periods of 24 hours or longer were necessary for the production of a fair amount of reducing sugars. In subsequent work the significance of this observation has not been sufficiently considered.

We have estimated the diastase content of dialyzed extracts of muscle, heart and liver of rabbits by determining the amount of glycogen which disappears during one hour of incubation at 37° C. Extracts from unperfused organs showed diastatic activity, but when the organs were perfused with Ringer's solution before the extracts were made, most of the diastatic activity disappeared. The same was true when press juices were prepared in a hydraulic press from ground rabbit liver mixed with diatomaceous earth and sand. Here again the diastatic activity was very low, if the liver had been perfused prior to the preparation of the press juice (3).

The diastase content of the blood of various species shows very marked variations; it is, for example, about 10 times higher in the blood of dogs, guinea pigs and rats than in that of rabbits. Since it is impossible to remove all the blood of an organ by perfusion, one would expect extracts of perfused rat liver to show a higher diastatic activity than that of rabbit liver. This was found to be the case, and in such extracts the phosphorylating activity to be described later was largely overshadowed by diastatic activity. These observations lead us to believe that an intracellular diastase, if it occurs at all in liver and other organs, cannot play a very important role in the breakdown of glycogen.

Phosphorylase. That a glycogen phosphorylating enzyme is present in muscle has been recognized for some time, but it is only recently that the activity of this enzyme has been defined, that the product formed by it has been isolated (4), and that it has been shown to occur not only in muscle but in other tissues (heart, brain, liver) as well (5). It is also found in yeast (5) and may be expected to be widely distributed in the animal and plant kingdoms.

When inorganic phosphate and a catalytic amount of adenylic acid are added to dialyzed extracts of muscle, heart, liver or brain, glycogen disappears very rapidly. The enzyme which causes this glycogen breakdown has been named (glycogen)-phosphorylase (6). When the addition of one of the components of the system, *i.e.*, either inorganic phosphate or adenylic acid, is omitted, the disappearance of glycogen is very small and corresponds roughly to the diastatic activity of the extracts. Phosphorylase catalyzes the following reaction in a reversible manner:

$$\text{Glycogen} + H_3PO_4 \rightleftharpoons \text{glucose-1-phosphate} \quad (1)$$

Other enzymes. Crude tissue extracts contain, beside phosphorylase, other enzymes the activity of which had best be considered before that of the phosphorylase is described. In all animal tissues so far investigated, and in yeast, there occurs an enzyme, phosphoglucomutase (7), which causes an intramolecular migration of the phosphate group from carbon atom 1 to 6:

$$\text{Glucose-1-} \rightarrow \text{glucose-6-phosphate} \quad (2)$$

Its concentration appears to be highest in skeletal muscle and is relatively low in brain and liver. Mg^{++}, Mn^{++} and Co^{++} ions markedly accelerate the activity of this enzyme. The optimal Mg^{++} concentration is 0.005 M, which is close to that found in muscle, while Mn^{++} and Co^{++} exert their optimal effect at 0.0005 M concentration. The shape of the curve of phosphoglucomutase activity in the presence of increasing concentrations of Mg^{++}, as well as the fact that the enzyme retains some activity in electrodialyzed extracts, suggest that it is not entirely inactive in the absence of specific ions. With the aid of radioactive phosphorus Meyerhof and collaborators (8) proved the correctness of the concept of an intramolecular migration of the phosphate group in reaction 2. It has not been possible to show a reversibility of the reaction catalyzed by the phosphoglucomutase, a point which deserves special emphasis and will be taken up again in the last section.

A third enzymatic process is catalyzed by the isomerase, an enzyme discovered by Lohmann (9) and shown to be present in animal tissues and in yeast:

$$\text{Glucose-6-} \rightleftharpoons \text{fructose-6-phosphate} \quad (3)$$

For this reaction neither coenzymes nor specific ions appear to be necessary.

The hexosemonophosphate isolated by Robison from the products of fermentation of glucose by yeast and from muscle by Embden was later shown to be a mixture of about 80 per cent of an aldose and 20 per cent of a ketose component. The structure of these two components has been definitely established as glucose-6- and fructose-6-phosphate. It was shown by Lohmann (9) that these two components, in the proportions mentioned, are the result of an enzymatic equilibrium. The enzyme is extremely active, so that equilibrium is reached in a few seconds following the addition of either glucose-6- or fructose-6-phosphate. This observation was of considerable importance, because it gave the first indication that reversible enzymatic equilibria play a role in lactic acid and alcoholic fermentation.

· With the formation of the equilibrium hexose-monophosphate the first stages of glycogen breakdown, common to both anaerobic and aerobic pathways, have been completed. The phosphorylation of fructose-6-phosphate to fructose-1-6-diphosphate and further anaerobic reactions terminating in the formation of lactic acid (or of alcohol in yeast) are described in the Embden-Meyerhof-Parnas scheme and will not be discussed.

Aerobically glucose-6-phosphate can be oxidized to gluconic acid-6-phosphate by a specific dehydrogenase for which triphosphopyridine nucleotide acts as coenzyme (10). The reduced coenzyme is oxidized by a specific enzyme and can thus react with molecular oxygen if suitable "carriers" (methylene blue, cytochromes) are present (11). By successive combination of the coenzyme with other catalytic proteins the oxidation can be carried to the 3-carbon stage. One may tentatively assume that the first branching of the ways occurs after reaction 3, one path leading to oxidation and the other to further anaerobic reactions. It was found (11a) in experiments on intact muscle that hexosemonophosphate disappears three times as rapidly under aerobic as under anaerobic conditions and that iodoacetate has no effect on the rate of aerobic disappearance of hexosemonophosphate. A branching of the ways at the glucose-1-phosphate stage would occur if this compound, by oxidation on carbon atom 6 and dephosphorylation, were shown to be the precursor of glucuronic acid. A discussion of oxidations at later stages of anaerobic breakdown will not be included.

Intact muscle. The formation of hexosemonophosphate by esterification of glycogen with inorganic phosphate was first discovered on intact muscle. The equilibrium hexosemonophosphate is a normal constituent of resting muscle and is the only intermediate between glycogen and lactic acid which accumulates in large amounts in a stimulated muscle. Up to 50 per cent of the glycogen broken down during a tetanus of 10 seconds duration may be present as hexosemonophosphate, the rest being present as lactic acid (12). A method for the determination of hexosemonophosphate in muscle which is in use in our laboratory consists in the separation of the monoester from other phosphate esters by fractionation as the barium salt and its determination by two independent analytical procedures, by its P content and its reducing power with an alkaline copper reagent (13). The use of this method was chiefly responsible for the detection of glucose-1-phosphate as the first product of glycogen degradation.

In order to study the mechanism of formation of hexosemonophosphate, it was desirable to find some method for increasing its concentration in muscle without causing at the same time an appreciable lactic acid formation. If both hexose-

monophosphate and lactic acid increase, as is the case following stimulation, it is impossible to say whether a given change in a phosphate fraction is related to one process or the other.

It was found that when thin frog muscles are kept anaerobically in Ringer's solution containing epinephrine in a concentration of 1 to 10 million, the hexosemonophosphate concentration rises markedly without more than a slight rise in lactic acid (14). This increase in hexosemonophosphate is accompanied by a decrease in inorganic phosphate without significant changes in other phosphate esters, *i.e.* phosphocreatine and adenosinetriphosphate. When the muscles, in addition, were poisoned with iodoacetate, the accumulation of hexosemonophosphate (corresponding to 8 mg. P per 100 gm. muscle) was accompanied by an equivalent decrease in inorganic phosphate (corresponding to 7 mg. P per 100 gm. muscle). Here again the changes in other phosphate esters were insignificant. By way of contrast lactic acid formation was increased without changes in hexosemonophosphate by addition of dinitrophenol or caffein to anaerobic frog muscle. In these instances phosphocreatine showed a decrease and inorganic phosphate an equivalent increase, while adenosinetriphosphate remained unchanged. When iodoacetate was added under these conditions, phosphocreatine decreased as before, but since fructosediphosphate accumulated instead of lactic acid, inorganic P was not liberated. It was pointed out that the decrease in phosphocreatine was due to its reaction with adenylic acid forming adenosinetriphosphate and that this reaction must have been preceded by one in which adenosinetriphosphate transferred its labile phosphate groups with formation of adenylic acid. Hence it was clearly established in these experiments on intact muscle that the formation of hexosemonophosphate involved only esterification with inorganic phosphate, while the formation of fructosediphosphate required the participation of adenosinetriphosphate as phosphate donor (15). Somewhat later Parnas and Baranowski and Ostern and Guthke (16) reached the same conclusions in experiments with muscle extract and they showed that adenosinetriphosphate reacts with hexosemonophosphate:

$$\text{Adenosinetriphosphate} + 2 \text{ fructose-6-phosphate}$$
$$= 2 \text{ fructose-1-6-diphosphate} + \text{adenylic acid}$$
$$(4)$$
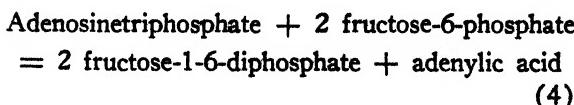
The trend among some workers in this field is to give more weight to experiments with muscle extract than with intact muscle, while others point out that what is found in muscle extract may be an artifact and may not apply to intact muscle. It may be, therefore, of some interest to refer to these experiments on intact muscle as well as to the sequence of observations in this field.

Minced muscle. Experiments were next carried out with minced frog muscle which was extracted three times with distilled water (4a). The purpose of this treatment was the removal of coenzymes and of organic phosphates which could act as phosphate donors. When the muscle residue which contained unextracted glycogen was suspended in phosphate buffer, inorganic phosphate disappeared and hexosemonophosphate was formed, while lactic acid formation was negligible. The effect of addition of coenzymes was tried and it was found that adenylic acid greatly enhanced the formation of hexosemonophosphate. After three hours of incubation most of the hexosemonophosphate formed was present as equilibrium ester, as shown by the equivalence of reducing power and organic P, but after short periods of incubation it was noted that much more organic P was present than corresponded to the reducing power. The compound formed during short periods of incubation was isolated from the muscle residue over the crystalline brucine salt. The analytical results for C, H, P, and Ba agreed with that of a hexosemonophosphate. Since the compound had no reducing power before, but yielded equivalent amounts of glucose and inorganic phosphate after acid hydrolysis, it was assumed to be glucose-1-phosphate. The high specific rotation ($[a]_D = 120°$ for the free acid) suggested a-configuration and thus related the compound to the a-glucosidic chain of glycogen. Synthesis of the compound by interaction of acetobromoglucose and silver phosphate, followed by the splitting off of two sugar residues and deacetylation, established the structure as a-glucopyranose-1-phosphoric acid. The synthetic compound was identical in its chemical and biological behavior with the natural product (17).

When the new ester was added to a freshly prepared muscle extract (or, as recently shown by Holmes (18), to tumor hash) it was converted to lactic acid; when added to a dialyzed muscle extract it was converted to the equilibrium ester. The latter conversion occurred at a much slower rate in the extracted muscle residue than in muscle extract. It is now clear why the muscle residue was a particularly useful preparation. Besides removing Mg^{++} ions, the water extraction removed more of the phosphoglucomutase than of the phosphorylase, thus creating conditions which permitted the glucose-1-phosphate to accumulate.

Tissue extracts. Ostern and collaborators (19) from Parnas laboratory reported shortly afterwards that a coenzyme was not necessary for the formation of equilibrium ester from glycogen and inorganic phosphate in dialyzed and aged muscle extract. In a later paper Parnas and Mochnacka

(20) confirmed the activating effect of adenylic acid in more thoroughly dialyzed extracts and they stated that inosinic acid acted in a similar manner. Kendal and Stickland (21) and Bauer and collaborators (22) also confirmed the "cophosphorylase" activity of adenylic acid, while they found inosinic acid to be slightly active or inactive.

Parnas coined the term "phosphorolysis" by which is meant the disruption of the glycogen molecule by esterification with inorganic phosphate. The process becomes clear if one considers the mechanism of formation of glucose-1-phosphate which is illustrated in the following diagram.

CH₂OH ... HO-P-OH ... CH₂OH ... HO-P-OH
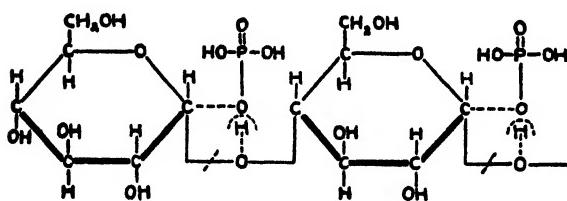
Inorganic phosphate takes the place of water and forms an ester linkage on carbon atom 1 of each glucose unit. The glucosidic linkage is thereby disrupted and the entire glycogen molecule is broken down into uniform fragments which consist of glucose-1-phosphate. If one measures simultaneously in an enzyme solution the disappearance of glycogen and the formation of glucose-1-phosphate, one finds that the two values agree closely (6). Although intermediates between glycogen and glucose-1-phosphate could not be detected in this manner, it is possible that depolymerization precedes the disruptive phosphorylation.

In the method for the determination of glucose-1-phosphate the ester is isolated as the water-soluble barium salt and hydrolyzed in weak acid to glucose and inorganic phosphate. This permits the determination, in the same solution, of glucose-6-phosphate which reduces alkaline copper solution directly and is not hydrolyzed under the conditions employed. With this method the formation of glucose-1-phosphate was demonstrated in dialyzed extracts of muscle, heart, brain, liver and yeast (5). The purpose of the dialysis was the removal of Mg^{++} ions which accelerate reaction 2, the conversion of glucose-1- to glucose-6-phosphate. A method for the isolation of glucose-1-phosphate has been described (17) in which about 0.5 gm. of the barium salt is obtained when 100 cc. of dialyzed muscle extract are used. Kiessling (23) described another method for the enzymatic preparation of glucose-1-phosphate in which the ester is obtained as the crystalline dipotassium salt.

The formation of glucose-1-phosphate in muscle extract escaped attention, partly for analytical

reasons and partly because the first point of action of Mg^{++} ions was unknown. Lohmann (24) had concluded that these ions are necessary for the esterification of glycogen. Subsequent experiments were generally carried out in the presence of Mg^{++} ions, i.e. under conditions which are unfavorable for the accumulation of glucose-1-phosphate. Since this ester is formed in muscle extracts which had been subjected to prolonged electrodialysis, Mg^{++} ions are not necessary for the esterification of glycogen, nor do they participate in the reversal of the reaction, the synthesis of glycogen from glucose-1-phosphate.

Adenylic acid. That adenylic acid (adenine-ribose-5-phosphoric acid) is essential for reaction 1 to go to the right follows from the fact that enzyme solutions can be prepared by prolonged dialysis which are inactive without addition of adenylic acid (25). When increasing amounts of adenylic acid are added to dialyzed muscle extracts, a typical coenzyme-activity curve is obtained. In enzyme solutions free of adenylic acid deaminase a definite effect is obtained with 3×10^{-5} M and an effect approaching a maximum with 10^{-3} M adenylic acid. A curve of similar shape is obtained with adenosinediphosphate, but this substance is only one half as active as adenylic acid. Inosinic acid in 2.5×10^{-3} M concentration has about the same effect as 6×10^{-5} M adenylic acid and a further increase in the inosinic acid concentration does not increase its effect. Adenosinetriphosphate is inactive in enzyme solutions which do not contain pyrophosphatase; if this enzyme is present it forms adenylic acid from adenosinetriphosphate. Yeast adenylic acid (adenine-ribose-3-phosphoric acid), cozymase, adenosine and muscle adenylic acid with the amino group combined with H_3PO_4 showed no coenzyme activity. Since adenosinetriphosphate is inactive and since it was found that adenosinediphosphate does not act by donating its mobile phosphate group, one may conclude that the transfer of inorganic phosphate to glycogen does not involve the reaction adenylic acid $+ 2 H_3PO_4 \rightleftharpoons$ adenosinetriphosphate.

Adenylic acid is also necessary for reaction 1 to go to the left. The purified phosphorylase (see section on glycogen synthesis), prepared from dialyzed extracts of muscle, heart and brain, does not form a polysaccharide from glucose-1-phosphate unless a catalytic amount of adenylic acid is added. Inosinic and adenosinetriphosphoric acids cannot be substituted for adenylic acid. Some difficulty was experienced with liver extracts, since they cannot be effectively dialyzed without a marked loss of phosphorylase activity, while purified enzyme preparations made from undialyzed extracts show considerable activity without addition of adenylic acid. It was possible, however, to

dialyze the glycerophosphate elution of the liver phosphorylase and to obtain an enzyme preparation which was barely active without and greatly activated by adenylic acid addition. It may be concluded that adenylic acid is the coenzyme of mammalian (glycogen)-phosphorylase and is necessary for the activity of the enzyme in both directions.

Effect of inhibitory and other substances. Lundsgaard (26) showed that phlorhizin inhibits lactic acid formation from glycogen in minced muscle by preventing phosphorylation. Ostern *et al.* (19) found that phlorhizin prevents the formation of equilibrium hexosemonophosphate in dialyzed muscle extract. Experiments in which the disappearance of glycogen and the formation of glucose-1-phosphate were determined showed that 0.01 M phlorhizin is an almost complete inhibitor of phosphorylase activity. Phloretin (the aglucone) has the same effect (6). Lehmann (27) showed that glucose inhibits the phosphorylase; the inhibition with 0.03 M glucose was about 50 per cent. Maltose, fructose, mannose, galactose, and glyceraldehyde, according to our experience, have no inhibitory action. Iodoacetate, NaF and KCN do not inhibit the phosphorylation of glycogen (6).

Liver. The presence of the phosphorylase in liver extracts (5) immediately suggested that this enzyme may play a part in blood sugar formation, the more so, since both the low concentration of diastase in the liver as well as its kinetics argued against its being the enzyme responsible for blood sugar formation (see section on diastase). It was shown that when hexosemonophosphate is added to liver extract, it is split to glucose and inorganic phosphate. It was therefore proposed that blood sugar formation is due to a combined action of phosphorylase and a phosphatase (28). In balance experiments with liver extract it was shown that the glycogen which disappeared was at all periods of observation accounted for by the sum of hexosemonophosphate and fermentable sugar (glucose) formed. When the enzyme solutions were incubated with glycogen and adenylic acid but without addition of inorganic phosphate the disappearance of glycogen was only a small fraction of that observed in the presence of phosphate. It was possible to separate the phosphorylase from the phosphatase by adsorbing the former on aluminum hydroxide. In the enzyme solution obtained by elution of the adsorbed phosphorylase the glycogen which disappeared was balanced by hexosemonophosphate formation, and no fermentable sugar appeared (3). Ostern and Holmes (29) recently reported that they have also obtained evidence for the predominant role of phosphorylase and phosphatase for the formation of blood sugar in the liver.

Glycogen synthesis. In order to demonstrate the reversibility of reaction 1 it is necessary to separate the phosphorylase from enzymes which remove glucose-1-phosphate, *i.e.* from phosphoglucomutase in muscle extracts and from both phosphoglucomutase and phosphatase in liver extracts. A method for the purification of the phosphorylase, applicable to all tissue extracts, consists in adsorption of this enzyme on aluminum hydroxide gel, elution with sodium glycerophosphate and precipitation of the elution with 0.3 saturated ammonium sulfate. The ammonium sulfate precipitate is dissolved in water. When glucose-1-phosphate is added, inorganic phosphate is liberated and a polysaccharide is formed. When this polysaccharide is determined according to Pflüger's method for glycogen, the values agree closely with those calculated from the inorganic P liberated. Mannose-1- and galactose-1-phosphate (30) are not converted to a polysaccharide by the phosphorylase.

Polysaccharide synthesis has been demonstrated with enzyme preparations from yeast (Kiessling, 31) and from mammalian muscle (32), heart, liver and brain (3); in all these cases the reverse reaction had previously been observed (5). The polysaccharide synthesized by the liver enzyme (prepared from rabbit, cat, and rat liver) is indistinguishable from glycogen in all properties so far investigated. It forms an opalescent solution in water, it is not destroyed by strong alkali at 100° C., it is precipitated by 50 per cent alcohol, it does not reduce alkaline copper solution and it is split to glucose by acid hydrolysis. It gives the same brown color with iodine as pure glycogen prepared from rabbit liver by the method of Somogyi (33). The polysaccharide formed by the muscle phosphorylase has the same properties except one; it gives a pure blue color with iodine. This color disappears on heating and reappears on cooling, exactly in the same manner as the blue color given by starch. The "blue glycogen" differs from starch by its solubility in cold water. Its molecular size is under investigation. Both types of polysaccharide, when added to dialyzed muscle extract with inorganic phosphate and adenylic acid, are converted to glucose-1-phosphate. The polysaccharide formed by the heart enzyme gives a brownish-purple color with iodine, that by the brain enzyme a reddish-purple color.

While the brown color with iodine is regarded as typical for glycogen, there exists, interestingly enough, an account by Bernard (34) in which he describes "blue glycogen" prepared from denervated, previously fatigued rabbit muscle. Young (35) has not been able to duplicate this observation, but Bernard unfortunately does not describe the exact experimental conditions. Bernard also frequently mentions glycogen preparations which

gave a purple color with iodine and Young describes purified muscle glycogen which gave a brownish-red to reddish-violet color. Since it is unknown what configuration of the molecule is responsible for these color reactions with iodine, it is impossible to state what chemical differences exist between brown, purple and blue glycogen.

A marked difference was found between the kinetics of the liver phosphorylase on the one hand and the muscle, heart and brain phosphorylase on the other hand. With the liver enzyme the activity starts immediately upon addition of coenzyme and substrate, and the same equilibrium is reached with different enzyme preparations provided they are free of phosphoglucomutase and phosphatase. The polysaccharide synthesis (in enzyme solutions originally free of inorganic P) stops when 84 per cent of the added glucose-1-phosphate is split, that is, at equilibrium the ratio inorganic phosphate/glucose-1-phosphate equals 5.1 at 30° C. Kiessling (31) reported an equilibrium constant for the yeast enzyme of 5.2 at 30° C. The striking difference shown by muscle (and heart and brain) phosphorylase is the invariable presence of a lag period which may vary from a few minutes to many hours. It seems that the reaction during the lag period proceeds at an infinitesimal rate. The lag is followed by a sudden outburst of activity. (Since this was written it has been established that all preparations of liver enzyme contain traces of glycogen and that addition of traces of glycogen (0.5 to 10 mg. per cent final concentration) to preparations of muscle, heart, and brain enzyme abolishes the lag period). An example of polysaccharide synthesis with liver, heart, and brain enzyme is given in Fig. 1.
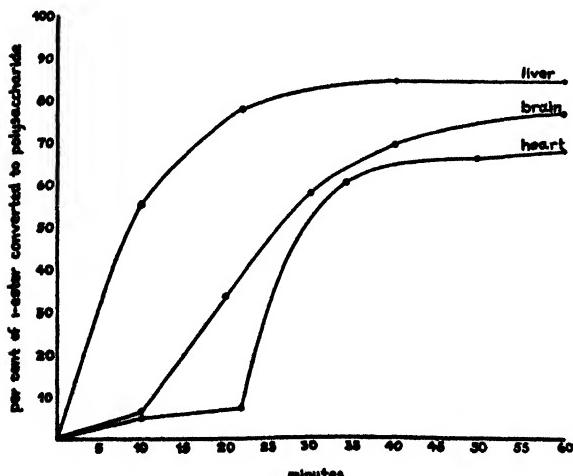
As long as no other mechanisms are known, it may be assumed that glucose must pass through a glucose-1-phosphate stage when it is synthesized to glycogen. One important problem is, therefore, the mechanism of glucose phosphorylation in animal tissues. It might be emphasized at this point that the glycogen phosphorylating enzyme discussed in this paper does not phosphorylate glucose.

Many animal tissues convert glucose anaerobically to lactic acid, but a clear case for a phosphorylated intermediate has not been made out Some authors actually believe that there exists a non-phosphorylating path for lactic acid formation from glucose. On the other hand, a clear-cut case of phosphorylation of glucose in kidney and liver pulp or extract under aerobic conditions has been established by Kalckar (36) and confirmed in our laboratory. Glucose in the presence of NaF is esterified with inorganic phosphate and is converted to fructosediphosphate which has been isolated. The first product of phosphorylation and the nature of the oxidations which are linked with the phosphorylation have not been established. If the first phosphorylation product should prove to be glucose-6-ester, its conversion to 1-ester (and hence to glycogen) would also depend on oxidative processes, since anaerobically the reaction glucose-1- → glucose-6-phosphate is not reversible. There exists some evidence in favor of such a hypothesis, since glycogen formation from 6-ester has been demonstrated in intact frog muscle under aerobic conditions (37).

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Fig. 1. Polysaccharide synthesis from glucose-1-phosphate with purified phosphorylase of liver, heart and brain.

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DISCUSSION

Dr. Bernheim: What is the function of the diastase in the blood?

Dr. Cori: It may not have a function there; it may represent an overflow of diatase from tissues in which it is produced. In diseases of the pancreas there is a very marked increase in blood diastase. A large amount of diastase is excreted in the urine. Blood diastase may originate in the pancreas and salivary glands and its fate may be excretion in the urine.

Dr. Nord: According to Deüticke and Hollman (Z. physiol. Chem. *258*, 175, 1939) the dissimulation of carbohydrates in tissues of ground organs passes mainly through the diphosphate phase, and in the intact muscle through the monophosphate phase. I wonder whether the experiments and the results presented are supposed to convey the idea that all the phases and preparations obtained from muscle extracts are necessarily the same also in the living, structurally unaltered tissue or cell.

Dr. Cori: The formation of hexosemonophosphate from glycogen and inorganic phosphate has been observed in intact muscle, in minced muscle and in muscle extract. I can conceive of only two reactions by which the large glycogen molecule can be broken down, by the introduction of the elements of water or of phosphoric acid. In the latter case glucose-1-phosphate is formed.

This intermediate cannot be demonstrated in intact muscle, because it is changed to glucose-6-phosphate as rapidly as it is formed. I did not say that all results which have been observed with muscle extract apply to intact muscle. I said that certain intermediate reactions cannot be studied on intact muscle and that one has to resort to simpler systems in order to investigate them.

Dr. Velick: In following the action of diastase it is comparatively easy to detect polysaccharide intermediates in the breakdown of glycogen to glucose. Is there any evidence of such intermediates in the breakdown of glycogen by phosphorylation?

Dr. Cori: It is possible that intermediates between glycogen and glucose-1-phosphate exist, but so far we have not been able to detect them. If one measures in the same enzyme solution the disapparance of glycogen and formation of glucose-1-phosphate, one finds that the two values agree within 5 per cent. Apparently phosphorylated intermediates other than glucose-1-phosphate, if they are formed, do not accumulate in sufficient amounts to be detected by this method.

Dr. Burk: Is it still possible that you might have glycogen intermediates or compounds of different complexity and molecular weight? I wonder whether you consider the "blue" glycogen different from the "brown" glycogen. If so it might be possible to regard the kinetic equilibrium you have described as between hexosemonophosphate, inorganic phosphate and (at least) two forms of glycogen, the "blue" and the "brown"; for a time "brown" glycogen breaks down and "blue" glycogen forms, even after there is no further change in the phosphate content.

Dr. Cori: It is quite possible that glycogen undergoes depolymerisation before it is phosphorylated and, conversely, polymerisation from smaller units when it is synthesized from glucose-1-phosphate. Without further investigation and molecular weight determinations we are not prepared to say what the relation of the "blue" to the "brown" glycogen is.

Dr. Barker: Are there any oxidative mechanisms coupled with these phosphorylative breakdowns of the resynthesis reactions, or will these go on in nitrogen?

Dr. Cori: The formation of glucose-1-phosphate from glycogen and the reverse reaction are anaerobic.

Dr. Barker: I am particularly interested in the iodoacetic acid experiment, since the amount used corresponds exactly with what we used in the mammalian muscle. We did not have the clear demonstration of oxidation of hexosephosphate, but we have found that glucose added as a substrate apparently is oxidized just as well by the iodoacetic acid poisoned muscle as by the unpoisoned one. What is your opinion as to how that glucose might be gathered up into the cycle?

Dr. Cori: In the experiments of Kalckar with kidney extract (which we have confirmed) glucose, in the presence of NaF, is esterified with inorganic phosphate and converted to hexosediphosphate, a reaction which occurs only in oxygen. A hexosemonophosphate might be expected to be the first product formed. The difficulty is that in Kalckar's experiments iodoacetate completely prevented the esterification of glucose. This leaves two possibilities, a mechanism for the oxidation of glucose which does not involve a phosphorylated intermediate or incomplete iodoacetate poisoning in mammalian muscle.

Dr. Burk: I think that the data offered here fit in very well with the work and conceptions of Engelhardt which I mentioned earlier in this Symposium. As Engelhardt has pointed out, the iodoacetic acid is not a (marked) inhibitor of oxidation of hexosemonophosphate: as long as one starts with glucose or glucosemonophosphate one can get unchanged oxygen consumption; the oxidation (but not glycolytic) utilization of hexosemonophosphate fits in very nicely with the observations of Warburg, Lipmann, Dickens, and now Engelhardt.

Dr. Lipmann: So far as I have understood, Cori says that he does not think that it is disproved that in the Pasteur effect the non-formation of lactic acid can be due to resynthesis. I think he will admit that the experiments of Dixon show that in the lactic acid respiring brain, no more lactic acid disappears than can be accounted for by respiration. They may not disprove resynthesis absolutely, but they show that starting with lactic acid no carbohydrate appears in brain. The great difficulty in disproving the resynthesis directly induced me to try to show experimentally that other possibilities exist in attempting to understand the Pasteur effect. I showed that an inhibition of glycolysis can be effected by oxidation. But these experiments are not sufficient to disprove the resynthesis theory; it would be necessary to make an experiment in which, with low respiration values in the intact cell, no glycolysis appears. I have tried to find some inhibitor which could do that, but I have not succeeded. In experiments with embryonic heart I found in two out of four experiments that malonic acid inhibited respiration, but glycolysis did not appear. Unfortunately, however, only in two of the experiments was this true; in the other two glycolysis appeared and therefore the experiments were never published. So long as such an experiment is not performed the possibility exists that resynthesis can take place. But I cannot understand why the cell expends such a large amount of energy to retransform an intermediate product to its parent material, because a mechanism exists which can inhibit the formation of this product from the parent material.

Dr. Cori: I would like to enlarge upon one short statement, made in my paper, which may have been misunderstood. I expressed the opinion that lactic acid can go back to glycogen in muscle by a reversal of the enzymatic processes which led to its formation and that it does not appear to be justified to call a synthetic process for which oxidative energy is required a Pasteur effect. I agree with Lipmann that if one finds an inhibition of lactic acid formation in oxygen, the lactic acid missed is probably never formed. I was referring to experiments with muscle in which lactic acid itself was used as substrate. You may recall that in Meyerhof's experiments lactic acid was actually shown to be present in muscle either as the result of stimulation or by adding it from without, and that a large amount of lactic acid disappeared which was not accounted for by the oxygen consumption. We reported experiments with thin frog muscle in which a resynthesis of glycogen occurred in two to three hours after stimulation instead of in twenty-four hours as in Meyerhof's experiments. Glycogen synthesis from lactic acid has also been demonstrated in the liver but according to Dixon it apparently does not occur in brain. It seems to me unjustified to assume an aerobic resynthesis of glycogen from lactic acid if the latter is merely assumed to have been formed because it appeared anaerobically and if the former is not actually determined, but this does not contradict the synthesis experiments in which lactic acid was used as substrate. These two experiments are not comparable and should not have been combined, as was done by Warburg when he spoke of a Pasteur-Meyerhof effect in all tissues.

Dr. Burk: I think we must always regard it as a fact, as Meyerhof really first showed, that lactic acid may be synthesized to glycogen under some conditions, as in the various experiments which Cori mentioned and in his own. The question in regard to Meyerhof's cycle theory, however, was not the qualitative conversion but the quantative conversion in relation to oxygen consumption. He once obtained a ratio of one to one of lactate disappearing to glycogen formed, but even here the "lactate oxidized" was of the same order, not one third to one sixth where a slight difference of opinion occurs. We must certainly recognize that lactic acid can be synthesized to glycogen; the major question now concerns the quantities involved; how much, in various cases, goes back to glycogen and how much to other materials.

Dr. Cori: I agree entirely with the idea that the lactic acid on its way to glycogen may be diverted in various ways and converted to other products. The Meyerhof quotient was used by Warburg in a slightly modified form as the basis of a general Pasteur effect and I wonder whether this has not created a great deal of confusion.

Dr. Burk: I think it is fair to point out with

regard to Warburg that he was invariably most wary of accepting the Meyerhof cycle; I do not believe one can find a statement of his accepting or applying it, but one can find several in which he took the trouble to point out that the Meyerhof quotient was one thing (a matter of experimental fact) and the Meyerhof cycle theory still another (a particular interpretation).

Dr. Cori: Yes.

Dr. Shorr: I am sorry if I seemed to infer in my paper that I doubted the existence of resynthesis. I was referring to the absence of adequate experiments on mammalian muscle and recognize that even in the absence of evidence of synthesis from the simple type of balance experiment ordinarily employed we could not infer, in view of the various possible intermediaries, that we did not have some type of resynthesis going on.

THE ROLE OF OXYGEN TENSION IN BIOLOGICAL OXIDATIONS

WALTER KEMPNER

When I was working on the problem of filtrable virus diseases in the Warburg Institute (1926-27), I found that the plasma of chickens infected with plague virus showed a particular kind of respiration which at that time I interpreted as due either to the metabolism of the plague virus itself or to a metabolism originating under the influence of the virus (1). The plasma of normal chickens did not respire, but Warburg (2) was subsequently able to produce a respiration in the plasma of chickens by prolonged asphyxiation, or by keeping them at a temperature of 37°C. for 90 minutes after death. The respiration in the plasma of plague virus chickens, the magnitude of which is approximately such that 1 cc. of plasma consumes in air as many mm^3. oxygen as 600 million fowl erythroblasts, with an R.Q. of 0.85, was insensitive to cyanide and carbon monoxide, and, moreover, it could not be inhibited by octyl alcohol or urethanes. I wrote in this paper that in other respects as well, the virus-plasma respiration was different from the respiration of cells and cell extracts. For I had found that this respiration was *markedly dependent* upon oxygen tension (Table I) and in general, and especially in the

TABLE I

Effect of various oxygen concentrations on the respiration of 3 cc. of plasma of a chicken infected with plague virus. 40° C.

Volumes p.c. of oxygen	4	20	50	100
$mm.^3$ of oxygen consumed	4.5	36.3	63	84.6

Warburg Institute, it was considered an unquestioned dogma that cell respiration is entirely independent of variations of oxygen tension (oxygen partial pressure, oxygen concentration—I use these terms interchangeably here) and continues at its optimal rate as long as the smallest *amount* of oxygen is still available. Warburg had apparently proved this in his experiments on sea urchin eggs (3) and on red blood cells of geese (4), and Oppenheimer had formulated this viewpoint in his handbook "Die Fermente" (5) in the sentence: "All observers agree that there is no true connection between the real oxygen tension with which the cell is actually in equilibrium and the rate of respiration." Earlier experiments on the subject, made before the introduction of the quantitative cell physiological methods, were criticized (4,5,6,7,8,9) as being vitiated by technical errors which always occur when the oxygen dif-

fusion cannot be ruled out as a limiting factor, *i.e.* in all those cases where only the surface of the liquid or of the tissue suspension is in equilibrium with oxygen of definite concentration, and not the surface of each individual cell.

The technical error of insufficient oxygen diffusion could easily be excluded in the experiments with virus plasma respiration; that the plasma was actually in equilibrium with the gas space was shown by altering the shaking speed of the manometer vessels, and the error of insufficient intracellular oxygen diffusion need not be considered here, since the respiration of this filtrable virus plasma is not bound to cellular structure, as is proved by its indifference to narcotics.

The rate of respiration in the plasma of plague virus chickens was essentially dependent on oxygen tension, and varied greatly with variations of oxygen tension not only between 4 and 20 volumes per cent, but also between 20 and 100 volumes per cent, similar in this to the rate of oxygen consumption of yeast maceration juice and of acetone yeast, which according to Meyerhof (10) at variations from 20 to 100 volumes per cent O_2 increases by 30-35 per cent. The increase of the plasma respiration rate at 100 volumes per cent O_2, as seen in Table I, was more than 130 per cent, the inhibition at 4 volumes per cent O_2 was 88 per cent (11). So it had been found that oxidation catalysts, separable from the cell and dependent on oxygen tension, may occur in the animal organism under certain conditions. But since this phenomenon of dependence on oxygen tension could not be reproduced in any of the numerous animal cells and bacteria examined then and previously, we had to assume, as Meyerhof did with regard to the yeast juices, that this plasma respiration was fundamentally different from cell respiration and represented the reaction of a particular kind of respiration ferment to which no significance attached in the respiration of animal cells and bacteria.

Today we know considerably more about oxidation catalysts that can be separated from the cell, since Warburg discovered and isolated the yellow ferments and, as he showed in 1932 (to anticipate the exact chronology), the rate of the isolated yellow ferment is very markedly dependent on variations of oxygen tension; at 760 mm.Hg the respiration rate was 4.8 times as high as at 38 mm.Hg (12). In the same paper, however, Warburg emphasizes again that the yellow ferment plays no decisive role in catalyzing respiration of cells.

As to cellular respiration, it is true that Meyerhof and Burk (13), Meyerhof and Schulz (14),

Burk (15) and Iwasaki, also in Meyerhof's Institute (16), described an exception to Warburg's law of independence of cell respiration on oxygen tension: the nitrifying bacterium *Azotobacter* which showed at 2 volumes per cent oxygen a decrease in the rate of respiration of 50 per cent compared to the rate of respiration in air. But the authors considered this result as an exceptional finding, valid only for *Azotobacter,* and so Meyerhof (8) quoting the above experiments wrote (1930) in a discussion of the oxygen requirements of the isolated muscle: "In the given formulae it is assumed that the rate of respiration of cells is independent of oxygen tension. This assumption corresponds to the general experience with the respiration of animal organs."

The "general experience" was Warburg's earlier work on sea urchin eggs (3) and red blood cells of birds (4), and mainly his experiments on "Respiration at Very Low Oxygen Tensions" (1929) in which he examined the respiration of *Micrococcus candicans* (17). Insufficient oxygen diffusion to the cell surface was excluded as a limiting factor. Warburg rotated the manometer vessels containing the bacteria suspended in Ringer-phosphate solutions in a water bath of 1°C. at a very high shaking speed, and found no difference between the rate of oxygen consumption in air and at 10^{-5} atmosphere. The low temperature was used for two technical reasons: first because at a low temperature the suspension fluid, due to the increase of the oxygen solubility, contains a greater amount of oxygen than at the same oxygen tension at higher temperatures, secondly, because, through low temperatures, growth of the bacteria during the course of the experiment can be prevented, and therefore one can be sure of working with a constant number of cells.

On the other hand, contrary to Warburg's all-or-nothing law of cellular respiration, a series of cells (18, 19), *e.g. Paramecia* and *Arbacia* eggs (20-23), yeast cells (16, 17, 24), luminous bacteria (25), *Sarcina lutea* (26), *Chlorella* (27) showed a certain dependence of respiration on oxygen tension. It must be noted, however, that some of these cells were of relatively large diameter and the effect of the variation of oxygen tension on respiration became evident only below 1 volume per cent of oxygen in the gas space of the manometer vessel, so that Gerard, who with his collaborators was the author of many of the papers just mentioned, concluded that it is impossible to decide how much of the inhibition found is due to the effect of oxygen tension on cellular respiration as such, and how much to insufficient oxygen diffusion (28). Even at last year's meeting of the American Physiological Society, in the symposium on anoxia, Gerard emphasized again that in no way need oxygen tension be considered as a limiting factor of cellular respiration.

This was also a tenet of our laboratory when in 1931 R. Lohmann (29) examined the sensitivity of malignant tumor cells to oxygen tension and found a marked inhibition of the respiration in sarcoma slices at 3.3 volumes per cent of oxygen compared to their respiration in air. Convinced of the evidence of Warburg's *Micrococcus candicans* experiment (17) which we cited as the crucial one, we reported our contradictory result with the comment that in experiments with tissue slices, no matter how thin they be, the factor of insufficient oxygen diffusion cannot be excluded.

Even when in experiments with isolated cells of very small diameter (pneumococcus) we did find a dependence of respiration on variations of oxygen tension (30)—and indeed to such a degree that at 8 volumes per cent of oxygen the respiration was considerably inhibited and at 2 volumes per cent the respiration was only 25 per cent of the respiration in air—we interpreted this as an exceptional case which we tried to harmonize with Warburg's results by explaining the sensitivity to variations of oxygen tension in pneumococci as a specific characteristic of bacteria whose respiration is catalyzed not by the iron-containing respiration ferment phaeohaemin, but by the yellow ferment; on the other hand, Bertho and Glueck (31), varying the oxygen tension only between 10 and 100 volumes per cent, had not been able to find dependence of respiration on oxygen tension in other bacteria with yellow ferment respiration, *Bacillus acidophilus* and *Bacillus acidificans Delbrückii.* That the respiration of animal cells and of bacteria with iron-ferment respiration was independent of oxygen tension, we then still accepted as an indubitable fact notwithstanding the results with pneumococci.

Since the question of dependence of respiration on oxygen tension is a key-problem whose solution is of basic importance, not only for the physiology of respiration, but also for the relation between respiration and fermentative splitting processes in the cell (Pasteur reaction), it is quite understandable why Warburg repeatedly (32, 33) resumed the subject to confirm his theory (1931-1935). The most recent occasion for this was the statement of Bumm, Appel and Fehrenbach (1934) that although respiration is independent of oxygen tension, aerobic lactic acid formation is not (34), a result which was also reported by Laser (1937) on the basis of experiments with retina and chorion (35). The material of Bumm and his co-workers was the mucous membrane of guinea pig intestine, the respiration and aerobic lactic acid formation of which they examined in

sodium chloride phosphate and in Ringer bicarbonate respectively, at 10 and 100 volumes per cent O₂.

The Warburg Institute replied to these experiments, in a paper by Leiner (33) to the effect that respiration and aerobic glycolysis of the guinea pig membrane had not been determined in the same suspension medium; also, that with tis· sue slices the factor of insufficient diffusion can· not be ruled out, and only isolated cells are suitable material for experiments on oxygen tension sensitivity. Leiner used cells with a very high respiration rate, the erythrocytes of rabbits poisoned with phenylhydrazine (Morawitz cells) (36), and examined them at optimal shaking speeds at 38°C.; small numbers of cells were suspended in sodium chloride bicarbonate solution. Aerobic lactic acid formation and respiration were measured manometrically at 5 to 6 volumes per cent of oxygen and in air. The rate of respiration as well as the rate of aerobic lactic acid formation remained unchanged regardless of the oxygen tension applied.

In the experiments with pneumococci (30) we had noticed that the dependence of respiration on oxygen tension is the greater, the younger the bacterial cultures that are used. In 48 hour old cultures examined in salt solutions not only was the absolute magnitude of respiration referred to dry weight of bacteria considerably decreased, but it was also hardly possible to find any dependence on oxygen tension. On the other hand the experiments with young pneumococci in a favorable culture medium were technically more difficult because, due to the handicap of possible growth, only short measuring periods were at our disposal, and there was always the danger that inhibiting growth, through lowering the temperature or diluting the suspension fluid with salt solutions without oxidation substrate, would at the same time create environmental conditions no longer optimal for the chemical reactions of the bacteria.

In view of these difficulties we began to examine systematically (37-45) a large number of young and old bacteria cultures in states of resting and growing, as well as isolated animal cells, tissue slices, tissue cell fragments and plant cells, and measured the effect of variations of oxygen tension on cellular metabolism under the various physiological and unphysiological conditions which we thought might influence the sensitivity of the reaction. The cells examined were red blood cells of fowls and cold-blooded animals, human erythroblasts and erythrocytes, mature and immature lymphatic and myeloid leucocytes, Morawitz cells of rabbits, kidney tissue slices and kidney cell fragments of rats and rabbits, tubercle bacilli, pneumococci, *Micrococcus candicans,*

Staphylococcus aureus, Escherichia coli, Pseudomonas pyocyanea, Monilia albicans and young pine needles.

Oxygen consumption and aerobic and anaerobic lactic acid formation were determined by Warburg's manometrical methods (6, 32), the chemical determination of lactic acid formation was done according to Fuerth, Charnas and Clausen with the modification of Friedemann and his coworkers (46-48), and the determination of amino acids in the experiments with kidney cells according to Van Slyke's ninhydrine method with the manometrical modification of Schlayer (49).

We took as our material first those cells with which Warburg had given experimental proof of his theory of independence of respiration on oxygen tension; we examined red blood cells of geese, Morawitz cells of rabbits and cultures of *Micrococcus candicans*, all under conditions identical with those of Warburg: *Micrococcus candicans* and bird erythroblasts at 1°C.; the Morawitz cells at 38°C. Our results were in complete agreement with Warburg's. The Morawitz cells examined at 38°C. at oxygen tensions between 3.8 and 20 volumes per cent as well as the blood cells of geese and the cultures of *Micrococcus candicans* examined at 1°C. at oxygen tensions between 1 and 20 volumes per cent and 0.1 and 20 volumes per cent respectively, showed no sensitivity to variations of oxygen tension.

But the question still remained whether these results necessarily had to lead to the conclusion Warburg drew from them, *i.e.,* that they were definite proof for the theory of the independence of cellular respiration of oxygen tension; or whether perhaps one might not consider inadequate an experimental set-up in which only abnormal cells were examined under physiological conditions, and normal cells only under unphysiological conditions.

Proceeding from our experiments with pneumococci where we had learned that older bacterial cultures are much less sensitive to oxygen tension than younger ones, we had some doubts whether the erythrocytes of rabbits poisoned with phenylhydrazine were exactly suitable material to solve the problem, in spite of the opinion expressed by Warburg (32) that "of all animal cells we know, the Morawitz cells are the best experimental material for respiration experiments"; because these cells which, like other mammalian erythrocytes, have lost their nuclei, must be regarded as senile and degenerated cells in a state of dying-off. For the purpose of controlling the methods to be applied, it was, however, of great advantage to work with such cells, for the fact that in spite of their high rate of respiration, one can submit them to very low oxygen tensions without finding any change in their respiration or lactic acid

fermentation, seemed to indicate that in the technique employed oxygen diffusion difficulties played no role. Young cultures of *Micrococcus candicans* and nucleated red blood cells, on the other hand, are a most suitable material, typical for undamaged isolated normal cells. But what seemed doubtful here was whether they could be expected to display all their characteristic metabolic reactions when examined only at a temperature lowered to 1° or 0°C.

Warburg's method of examining the bird erythroblasts was based on the fact that the affinity of hemoglobin to oxygen greatly increases with lowered temperatures. He wrote on this subject (4): "According to Barcroft and King (50), blood at low temperatures gives up the chemically bound oxygen only at very low oxygen tensions. For goose blood this 'dissociation tension' is at 0°C. below 5 mm. oxygen, *i.e.* at 5 mm. oxygen the hemoglobin-oxygen combination is but very little dissociated. If we then allow erythroblasts of geese to use their chemically bound oxygen at 0°C., we know that the oxygen is consumed at a lower tension than 5 mm."

In his experimental procedure, then, Warburg took into consideration the fact that the affinity of hemoglobin to oxygen is greatly influenced by low temperatures, but took it for granted that the oxygen affinity of the oxygen transferring ferment of cellular respiration was entirely independent of temperature. This, however, is by no means true. Rather is the dissociation of the oxygen combination of the oxygen transferring ferment of respiration so much influenced by temperature, that it was possible to overlook the whole phenomenon of dependence of cellular respiration on oxygen tension, if the experimental material, whether animal cells or bacteria, was examined only at temperatures of 1° or 0°C.

Examined under physiological conditions at 37° C., all nucleated uninjured blood cells and a great number of young bacteria, regardless of whether they are carbon monoxide and cyanide sensitive or not, *i.e.* whether their respiration is catalyzed by iron-containing phaeohaemin or by iron-free yellow ferment, show a decisive dependence of their respiration on variations of oxygen tension. For instance, the respiration of normal red blood cells of geese at 4.9 volumes per cent oxygen was inhibited by 49 per cent compared to that in air, and that of *Micrococcus candicans* at 2 volumes per cent was inhibited by 60 per cent. (Table II).

The shape of the oxygen saturation curve of the oxygen transferring ferment of respiration at various oxygen concentrations is shown by experiments with tubercle bacilli. Since they are relatively resistant organisms and their rate of growth is very slow, it is possible to examine them at 38°C. at a high shaking speed over a long period, up to 36 hours, and at the end of the experiment still to find a respiration rate in air practically identical to that at the beginning (43, 45). Therefore the same manometer vessel containing 1 to 2 cc. suspension fluid and 7 to 14 mg. bacteria can be successively saturated with many gas mixtures of varying oxygen concentrations without the bacteria showing either an irreversible decrease of their metabolism due to injury, or an increase due to growth. (Fig. I).

Most animal cells are, of course, more delicate, and it would be inadvisable to use them in this

TABLE II

Effect of lowered oxygen concentrations on respiration of various types of cells. 37.5° C.

Type of cells	Oxygen concentration (volumes per cent)	Inhibition of respiration (per cent)
Normal human erythrocytes	3.0	0
Morawitz cells of rabbits	3.6	0
Blood cells of normal goose	4.9	49
Blood cells of anemic goose	6.1	70
Blood cells of patient with erythroblastic anemia	4.9	57
Blood cells of patient with myelogenous leukemia	5.2	60
Human myeloblasts	7.9	48
Pneumococcus Type I	2.0	72
Micrococcus candicans	2.0	60
Staphylococcus aureus	3.4	60
Escherichia coli	3.4	50
Tubercle bacillus H 37	4.0	40
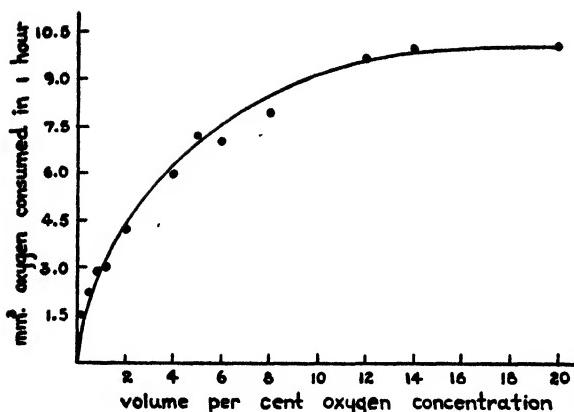
Fig. 1. Rate of respiration of 1 mg. dry weight of tubercle bacilli H 37 at various oxygen concentrations. 37.8° C.

kind of respiration experiment over periods longer than one or two hours. They are easily injured by cooling, centrifuging, high shaking speed, an-aerobiosis, even of short duration, and by substi-tution of salt solutions for their original plasma. Blood cells of geese with marked anemia proved the most suitable material, whereas red blood cells of turkeys, chickens, alligators and bullfrogs were less resistant, changing their metabolism more readily as shown by decrease of respiration and gradual appearance of lactic acid formation in air. Such injured animal cells lose their sensitivity to variations of oxygen tension just as do old bacteria cultures. Their absolute rate of oxygen con-sumption, though, may remain relatively high and constant for a long time, as for instance in ma-ture white blood cells of leukemic patients, but this oxygen consumption due to residual oxidative processes persisting or appearing after the cell has entered a state of dying-off, should not be mistaken for the true physiological cellular respir-ation, *i.e.* the oxygen consumption that is found in uninjured cells examined as soon as possible after being taken from the patient or experimental animal, and under conditions resembling those actually present *in vivo* as closely as possible. The unphysiological, though theoretically interest-ing, oxidative processes which outlast the perish-ing of cells and cell fragments under various artificial conditions *in vitro*, may prove indifferent to the influence of oxygen tension, but it would not be within the scope of this paper, which is exclusively concerned with the true physiological respiration, to discuss this indifference to varia-tions of oxygen tension in other types of oxidative reactions, were it not for the reason that it pro-vides another simple means of ruling out the error of insufficient oxygen diffusion, always to be sus-pected in experiments with lowered oxygen ten-sion. For the rate of oxygen consumption in the

same cell suspension of mature leukemic leucocytes showed, in the first hour after the cells were re-moved from the body, a marked dependence on variations of oxygen tension which, however, by the third hour of the experiment, had completely disappeared.

The decided influence of temperature on the sensitivity of bacterial respiration to oxygen ten-sion is demonstrated by Fig. 2. The curves show determinations of the respiration of *Escherichia coli* at different oxygen tensions at temperatures of 6.5°, 15.5°, 22.8° and 37.8°C. It is apparent that with lower temperatures the curves become steeper. At an oxygen tension *e.g.* of 0.9 volume per cent, the respiration is still the same as in air at 6.5°C., whereas at 37.8°C. it is inhibited by more than 50 per cent at an oxygen tension as high as 3.9 volumes per cent. At temperatures between 4°C. and 0°C. one must use extremely low oxygen tensions to find any inhibition of respiration at all.

Micrococcus candicans, even when very young cultures were used and examined in an otherwise physiological environment at 1°C., showed, as previously stated, exactly the same rate of respira-tion at 0.1 volume per cent oxygen and in air. At 38°C. it showed an inhibition of respiration of 60 per cent at an oxygen tension twenty times higher.
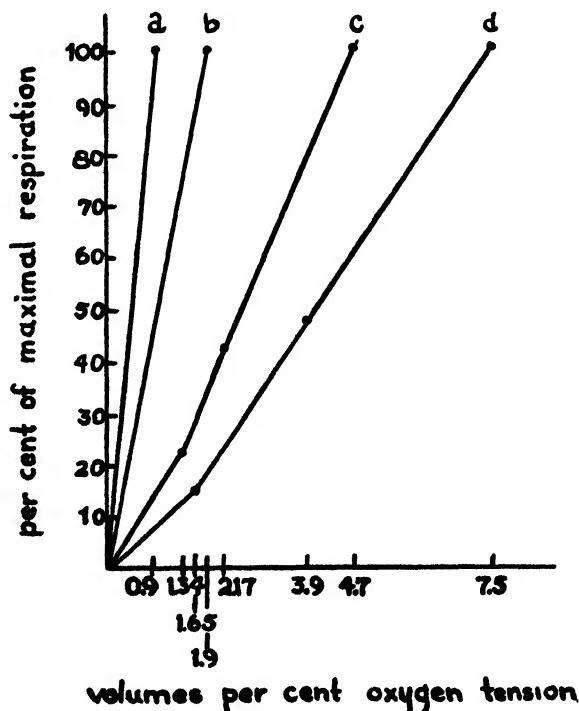
Fig. 2. Effect of temperature on sensitivity of respiration to variations of oxygen tension. *Escherichia coli.* (38) Curve a, 6.5° C. Curve b, 15.5° C. Curve c, 22.8° C. Curve d, 37.8° C.

The same sensitivity of the oxygen affinity of the respiration ferment to variations of temperature was seen in goose erythroblasts. At an oxygen tension of 3.8 volumes per cent the respiration at 38°C. was inhibited by 60 per cent as compared to air. At 26°C. the inhibition was only 22 per cent, at 10°C. no inhibition was found.

That the surface of the individual cell is really in equilibrium with the gas space of definite oxygen concentration; and that insufficient diffusion from the gas space to the cell surface is not a limiting factor in these experiments, is shown also by the controls with different shaking speeds, the experiments with mature leukemic leucocytes just mentioned, and by the experiments with the Morawitz cells. In the case of normal non-nucleated blood cells (human and rabbit erythrocytes) and of old bacterial cultures that also had proved insensitive to variations of oxygen tension, it might possibly have been said that here the oxygen tension effect failed to appear because the diffusion conditions were particularly favorable, since the absolute rate of respiration, referred to mg. cell weight per hour, was less than in nucleated cells or in younger bacterial cultures. This objection can be set aside, however, on the basis of the experiments with the Morawitz cells that have a respiration rate 40 times higher than normal rabbit and human erythrocytes, and 2 to 4 times higher than erythroblasts of geese.

Neither of these controls, however, can exclude the possibility of an insufficiency of intracellular diffusion. One could say that in non-nucleated cells the conditions for inner diffusion might for some reason or other be more favorable than in the nucleated cells, and that it was therefore fundamentally not the question of a *direct* effect of oxygen tension on the oxygen transferring ferment, but only of an *indirect* effect of decreased oxygen tension on the rate of respiration, in that the cell ferments, because of the blocking of the supply of oxygen required, were placed partly under aerobic, partly under anaerobic conditions, and that in this way decreased oxygen consumption was the final result. This objection could not be countered with controls obtained by applying even the greatest shaking speeds, as they would always merely concern the equilibrium between the cell surface and the surrounding medium, nor with the fact that cells like the experimentally damaged mature leucocytes, with a quantitatively constant but qualitatively changing respiration, or like the Morawitz cells, with a rate of respiration four times as high as that of the nucleated red blood cells of geese, are independent of variations of oxygen tension. Against this it could still be argued that the inner structure of these cells, and hence the intracellular diffusion conditions, were different, so that in one instance,

but not in the other, the necessary amount of oxygen reached the oxidation ferments at the right time. We would have been unable to decide this matter if the characteristic metabolism of the cells had not itself come to our assistance, and here again it was the nucleated blood cells, red blood cells of birds and cold blooded animals, human erythroblasts and white blood cells, that furnished excellent material, for in these cells we have a conspicuous indicator for the onset of anaerobic conditions at any time and place, namely the formation of large amounts of lactic acid. If, then, decreased oxygen tension inhibited the oxygen consumption because at some time and somewhere in the cell partial anaerobiosis occurred as the result of insufficient oxygen diffusion, this would have made itself manifest through increased lactic acid formation. As I have said, the cells were most cooperative in this whole matter, and without this cooperation it would not have been possible to give the desired proof with certainty, for it might well have happened that in the first place not only the complete absence of oxygen but even the lowering of the oxygen tension might have caused an increase in lactic acid formation, along with the inhibition of respiration, or secondly, that, as the Warburg-Meyerhof theory postulates (6, 7, 8) every decrease in respiration might have brought about a direct increase in the formation of lactic acid. The comparison with the non-nucleated Morawitz cells, experimentally damaged mature leukemic leucocytes, and the old bacteria cultures, as well as the independence of the results from shaking speed alterations, would perhaps have caused us to maintain our opinion that the phenomenon we had found was indeed due to the direct effect of oxygen tension on respiration, but we should not have been able to confirm this with quantitative data excluding the possibility of insufficient intracellular diffusion.

Fortunately the lowering of oxygen tension does not act reciprocally on respiration and lactic acid fermentation, *i.e.* lowered oxygen tension does not produce a corresponding increase in lactic acid formation simultaneously with the inhibition of respiration, nor do the nucleated blood cells react according to the Warburg-Meyerhof theory that lactic acid is formed whenever respiration is inhibited. Rather, it became evident that through lowered oxygen tension a great inhibition of respiration could be produced without any lactic acid formation occurring. In the manometrical determination, instead of a rise in the quotient (CO_2 formed /O_2 consumed) which must be expected when lactic acid is formed in a bicarbonate-containing medium, there was, on the contrary, a decrease of this quotient which, as seen in Table III and Table VI, proved to be a true decrease

TABLE III

Effect of lowered oxygen tensions on the metabolism of 100 mg. goose
erythroblasts in one hour. 37.8° C.

Volumes per cent of oxygen	mm.³ oxygen consumed	mm.³ carbon dioxide formed in respiration	R. Q.	mm.³ lactic acid formed*
19.3	65	58.5	0.9	6.6
7.7	65	58.5	0.9	6.4
5.9	57.5	49.7	0.865	6.9
4.9	33.2	6.7	0.202	6.2
3.8	22.8	0.59	0.026	5.9
1.6				21.0
0	0	0	0	75.0

* 1 mm.³ = 0.004 mg.

of the respiratory quotient, revealing that at lowered oxygen tensions the respiration of the blood cells was also qualitatively changed.

Along with the manometrical determinations we determined the lactic acid formation chemically as well, at many different oxygen tensions and under various conditions and, in complete agreement with the manometrical experiments, obtained the unambiguous result that through decrease of oxygen tension the respiration of nucleated blood cells can be notably decreased, for instance by 70 per cent compared to the respiration in air, and that, notwithstanding, the cells fail to react with any kind of lactic acid formation (Fig. 3).

We still offered ourselves the rather far-fetched objection that in bird erythroblasts washed in salt solutions and suspended in Ringer-bicarbonate, the finding of the same lactate content after exposure to low oxygen tension as after exposure to air could be due to the fact that the supposedly anaerobic parts of the cells might indeed form lactic acid, but that this lactate might act on the aerobic cell parts as a particularly stimulating and easily oxidizable substrate that would immediately be removed through oxidation, while cells kept in air would form no lactic acid and oxidize other substrates, so that the final result with regard to the lactate content would be identical under both conditions. If, however, the cells are not suspended in salt solutions but in plasma, this objection can be disregarded, since the normal lactate concentration of fowl plasma is so high to begin with (2 to 3 $\times$ 10^{-3} mol) that the increase, caused by the additional amount of lactic acid that might be formed, would be negligible. Besides, experiments in which erythroblasts were suspended in Ringer-bicarbonate solutions of different lactate concentrations gave no support to the assumption of varying substrate selection of these cells at varying oxygen tensions.

It is proved, then, by the fact that no lactic acid formation occurs at definitely low oxygen tensions in nucleated red and white blood cells, that one can considerably decrease the oxygen tension without producing any anaerobiosis within the cells; that is to say, that at low oxygen tensions, just as if the suspension were saturated with air or pure oxygen, not only the extracellular diffusion between gas space and cell surface but also the intracellular diffusion between cell membrane and chemical catalysts is sufficient to furnish the amount of oxygen required. Since, therefore, diffusion can be excluded as a limiting factor in these experiments, the decrease in cell respiration at low oxygen tensions can only be explained by a direct reaction of the oxygen of definite concentration with the oxygen-transferring ferment of respiration itself.

One more possibility of demonstrating that insufficient oxygen diffusion has no part in explaining the effect of oxygen tension on respiration was to change the chemical composition of the cell
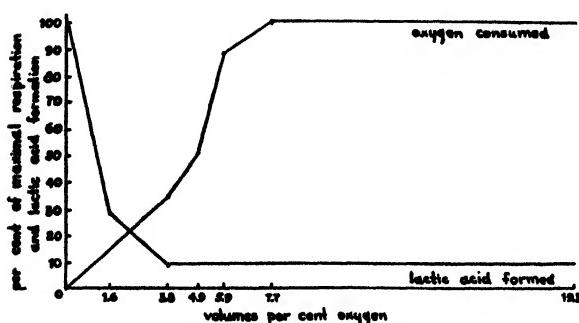
Fig. 3. Rate of respiration and lactic acid formation at various oxygen tensions. 37.8° C. (38).

milieu. The cells were either kept in their own plasma in the absence of carbon dioxide at an alkaline pH (NaOH in the side bulbs), or the physiological buffer-system bicarbonate-carbon dioxide was replaced by phosphate buffers of the same pH.

We know that the substitution of phosphates for physiological bicarbonate-carbon dioxide buffers is not an indifferent procedure but changes the course of reaction's in a number of chemical processes in the cell. This may be illustrated by three examples. Warburg had found (32) that carbon monoxide (5 volumes per cent CO_2/5 volumes per cent O_2/90 volumes per cent CO) inhibits the respiration of the Morawitz cells by 67 per cent, an inhibition he observed with the same proportion carbon monoxide/oxygen also in yeast. That this inhibition was really due to the effect of carbon monoxide and not to the effect of the low oxygen tension was proved by a control with 5 volumes per cent CO_2/5 volumes per cent O_2/90 volumes per cent argon. This inhibition of respiration by carbon monoxide did not occur when the cells were examined in sodium chloride-phosphate instead of in Ringer-bicarbonate/CO_2. Warburg communicated this result with the following comment: "Similar phenomena in other cells have been the cause of controversies." (He refers here to a paper of Dixon and Elliot (51) who had examined the influence of cyanide on liver and kidney slices suspended in sodium chloride-phosphate and had found in this milieu only an incomplete inhibition of the respiration of those cells.) "This may teach us that if animal cells show a different behavior in sodium chloride solutions, they must be examined in their physiological salt milieu."

Another instance is the behavior of the respiration of rat retina to cyanide, reported by Laser (52). In contrast to most other animal cells, rat retina suspended in Ringer-bicarbonate showed a respiration entirely uninfluenced by M/100 cyanide. In phosphate the same cyanide concentration inhibited the respiration by 90 per cent, although in Ringer-bicarbonate the absolute magnitude of respiration was even greater.

A third example is in the experiments of Friedheim (53) who showed that pyocyanine, in the presence of phosphates, causes considerable increase in the respiration of normal rabbit kidney cells, and that this does not occur in Ringer-bicarbonate. From this finding Friedheim concludes that "in phosphate solution the oxidation mechanism of the cell is fundamentally different."

There is no doubt that phosphate buffer solutions are unphysiological for animal cells, and it is difficult to compare the processes observed in such solutions with those in the living cell. It would,

however, be wrong, as Gaffron points out (54), "to set aside the results obtained in phosphate solutions for this reason, and to accord them no value in forming our ideas on the course of cellular reactions." At any rate, metabolism investigations in phosphate, in addition to those in serum-bicarbonate-carbon dioxide or Ringer-bicarbonate-carbon dioxide, are necessary contributions toward the explanation of the mechanism of cellular respiration and fermentation and the relationship of one to the other. But the experiments on dependence of respiration on oxygen tension teach anew that one should not perform experiments in carbon dioxide-bicarbonate-free sodium chloride or phosphate solutions before the fundamental facts have been established in a physiological suspension fluid, preferably in the animal's own plasma.

Our experiments with nucleated red blood cells show that the affinity of oxygen to the catalyst of cellular respiration is essentially changed, not only by an alkaline carbon-dioxide-free milieu, but by the substitution of phosphate buffer for the physiological bicarbonate-CO_2. Under these unphysiological conditions the respiration becomes so insensitive to variations of oxygen tension that, just as shown above for low temperatures, so also by working in phosphate instead of bicarbonate-carbon dioxide, the entire phenomenon of the dependence of cell respiration on oxygen tension was bound to escape observation. It was not the concentration of the phosphate, varied between M/15 and M/100, that was important, but rather the absence of the normal physiological composition of the suspension fluid. We found, *e.g.*, in sodium chloride-glucose-M/60 phosphate (pH 7.4) at 38°C., at an oxygen tension as low as .0.8 volume per cent, the respiration of erythroblasts of geese to be exactly the same as in air, while in sodium chloride-glucose-bicarbonate-carbon dioxide the inhibition of their respiration compared with that in air was of 60 per cent at an oxygen tension as high as 3.8 volumes per cent. The same differences were seen between the respiration of erythroblasts measured in alkaline carbon dioxide-free plasma and those measured in the unchanged physiological plasma containing 5 volumes per cent CO_2. (Table IV).

It is, however, not for the question of the influence of environmental factors on the mechanism of cellular respiration that we discussed these experiments here, but for the question of oxygen diffusion. Since the same cells were examined at the same shaking speed, in the same proportion of cell volume to fluid volume and of fluid volume to gas volume (4 cc. to 15 cc.), with only the suspension media differing, a simple technique was at hand for excluding differences in either ex-

TABLE IV

Percentage of maximal respiration of goose erythroblasts in suspension media of different composition at various oxygen tensions. 38°C.

Volumes per cent of oxygen	NaCl glucose M/60 phosphate pH 7.4	Plasma alkaline	NaCl glucose	Plasma
	Carbon dioxide free (KOH in side bulbs)		2.5 × 10⁻³ mol NaHCO₃/5 p.c. CO₂	
20	69	95	87	100
3.8	69	91	34	35
1.6	69			16
0.8	69			

tracellular or intracellular diffusion as a possible source of error. The role of oxygen tension as a *direct* determining factor in cellular respiration could thus again be definitely demonstrated.

Not all the cells which we examined were influenced in their sensitivity to oxygen tension by salt composition of the suspension medium, presence or absence of carbon dioxide, etc. We found, for instance, that a great number of bacteria, unlike the blood cells, showed a marked dependence of respiration on oxygen tension, whether bicarbonate-carbon dioxide or phosphate solutions were used. Also the sensitivity of the rate of deamination of amino acids in kidney cells toward variations of oxygen tension did not change whether the kidney slices or kidney cell fragments were examined in bicarbonate-carbon dioxide-containing, or in phosphate buffered, milieu.

The fact that the rate of respiration of nucleated blood cells examined under physiological conditions decreases considerably with lowered oxygen tension without any lactic acid being formed, was important because of the question of intracellular diffusion. We have to go back to this experimental result in considering the problem of the mutual relations between respiration and lactic acid fermentation. Since Pasteur's discovery that many cells form lactic acid in the absence of oxygen, not much advance has been made beyond the two experimental facts: first, that a great number of cells (most normal animal cells) show respiration and no lactic acid formation when examined in air or in pure oxygen, and second, that in the total absence of oxygen, *viz.* at an oxygen tension of zero, in nitrogen, hydrogen, carbon monoxide, argon, etc., when respiration, of course, is zero, lactic acid is formed in large amounts. These two facts have led to the conclusion that the occurrence of lactic acid formation is caused by a decrease in respiration, the assumption being that respira-

tion and lactic acid formation are reactions coupled in such a way that whenever respiration decreases, the cell tries to compensate, by an increase in lactic acid formation, for the amount of energy lost through the inhibition of respiration. A quantitative relation between the oxygen consumed and the lactic acid formed was shown in a number of cells (8), 1 molecule of oxygen causing 1 to 2 molecules of lactic acid to disappear.

The effect of cyanide and carbon monoxide on numerous cells seemed to give support to this interpretation (6, 7). For both in cyanide and in carbon monoxide, an increase in the splitting processes was always found together with the inhibition of cell respiration. If, for instance (39), erythroblasts of geese were examined at 5 volumes per cent CO_2/20 volumes per cent O_2 in 5×10^{-4} molar cyanide, and an inhibition of respiration of 62 per cent was found, the lactic acid formation which at the same oxygen tension in the absence of cyanide was zero, rose to 71 per cent of the maximal anaerobic fermentation. If, however, the same cells were examined in the absence of cyanide and respiration was decreased by exposing the cells to 6 volumes per cent oxygen instead of to air, then, in spite of a 70 per cent inhibition of respiration, no lactic acid formation appeared. (Table V).

If in both cases, in cyanide as well as at an oxygen tension of 6 volumes per cent one finds an inhibition of respiration of 60 to 70 per cent, but increased production of lactic acid only in the case of cyanide, one must discard inhibition of respiration as a determining factor in interpreting the mechanism of the Pasteur reaction and also that of the effect of cyanide. It then follows that the cause of the lactic acid fermentation which occurs in complete absence of oxygen is not that the cells no longer respire and therefore try to obtain their energy in another way, but that the

TABLE V

Respiration and lactic acid formation of 100 mg. of goose erythroblasts at low oxygen tensions and in cyanide. 37.8° C.

Volumes per cent of oxygen	Mol. HCN	Respiration in 1 hour. (mm.^{3})	Lactic acid formation in 1 hour. (mm.^{3})	Inhibition of maximal respiration. (p.c.)	Maximal lactic acid formation. (p.c.)
19.3	—	179.0	0	0	0
19.3	5×10^{-4}	68.3	61.7	62	71
6.1	—	53.0	0	70	0
0	—	0	86.8	100	100

lactic acid fermentation catalyst itself is directly or indirectly affected by the absence of oxygen; or *vice versa*, that the disappearance or non-appearance of lactic acid fermentation in the presence of oxygen is not due to Meyerhof's hypothetical resynthesis of 1 to 2 molecules of lactic acid through 1 molecule of oxygen consumed in respiration, but to the reaction of oxygen with the lactic acid fermentation catalyst itself, regardless of whether the oxygen is transferred by one of the catalysts of respiration (55, 56, 19) or by some other oxygen carrier in the cell. As shown by Fig. 3, a reciprocal relation between the oxygen affinity of the lactic acid fermentation ferment and the oxygen affinity of the respiration ferment does not exist.

On the other hand, if the inhibition of respiration through cyanide must be discarded as the cause of lactic acid formation in cyanide/air, then lactic acid formation in cyanide can only be explained by an effect of cyanide on the catalytic system of the lactic acid fermentation itself, occurring simultaneously with the inhibitory effect on the catalytic system of respiration or, as in some algae cells, even without it (57, 58). According to Warburg, the inhibitory effect of cyanide on respiration is due to a reaction of cyanide with the iron of one of the respiration catalysts, so that the combination of the respiration catalyst with oxygen is prevented. The effect of cyanide on lactic acid fermentation can be explained in the same way. Assuming that the lactic acid fermentation is inhibited under aerobic conditions by the reaction of one of the lactic acid fermentation catalysts with oxygen, the effect of cyanide on cellular metabolism would be due to the fact that not only the combination of oxygen with the respiration catalyst, but at the same time with the fermentation catalyst, is prevented.

Correspondingly the effect of other substances which change the reactions of cellular metabolism can be explained, *e.g.*, of carbon monoxide, ethyl cyanide, and phenosafranine. Carbon monoxide, which inhibits respiration and increases lactic acid formation would, like cyanide, act by displacing the oxygen from the respiration ferment system as well as from the ferment system of lactic acid fermentation; ethyl cyanide and phenosafranine, which increase lactic acid fermentation without inhibiting respiration, would act exclusively with the ferment system of lactic acid fermentation (39).

In the field of abnormal cellular metabolism, *e.g.*, that of injured animal cells and cancer cells, our ideas on the mechanism of the relations between oxidative and fermentative splitting processes must also be modified by the fact that respiration can be appreciably inhibited by a decrease of oxygen tension without a corresponding increase in lactic acid formation. As Warburg has shown (6), normal undamaged animal cells do not form any lactic acid in air, whereas in the metabolism of benign tumor cells and more so in that of cancer cells, under the same aerobic conditions, a great amount of lactic acid is formed. This aerobic lactic acid formation is, however, not only seen in malignant tumors. It may be produced by cyanide, carbon monoxide, ethyl cyanide, as mentioned above, as well as by various injuries leading to the gradual death of the cell, or in more sensitive organs, simply by keeping the tissues in salt solutions instead of in their own plasma. Warburg explained the "aerobic" lactic acid formation of such cells as being caused by the insufficiency of inhibited or injured respiration, no matter whether this inhibition or injury of the cellular respiration catalyst takes place within or outside the body.

The abnormal metabolism type of exudate leucocytes which show, besides a high rate of respiration, a high lactic acid formation in air, was explained in the same way: that the exudate leucocytes were cells already dying off *within* the body; that the first sign of the dying-off of cells was injured respiration, and that this injured respiration could no longer master the lactic acid

TABLE VI

Metabolism of 1 mg. (dry weight) of myeloblasts per hour at various oxygen tensions (44). 2.5×10^{-2} M $NaHCO_3$. 8.5×10^{-3} M glucose. $37.5°$ C.

Oxygen tension (mm. Hg)	Oxygen consumed (mm.³)	Carbon dioxide formed (mm.³)	R. Q.	Lactic acid formed (mm.³)	Inhibition of respiration (p.c.)
760-160	7.7	5.8	0.75	0	0
60	4.0	0.95	0.24	0	48
45	3.22	0	0	0	58
0	0	0	0	11.5	100

formed, so that a large amount of lactic acid appeared even in air.

It is true that mature exudate and blood leucocytes of normal individuals and of patients with leukemia have a high rate of lactic acid formation, not only in complete absence of oxygen, but also in air; it is true, as was seen in human lymphoblasts (59, 60) and myeloblasts (61), that very young leucocytes also show a high lactic acid formation in complete absence of oxygen; none, however, in air. But curiously enough, by considerably inhibiting the respiration of these immature white cells, the myeloblasts, by lowered oxygen tension, the metabolism type of the mature leucocytes could nevertheless not be produced (44), *viz.* if through decrease in oxygen tension the respiration of the myeloblasts was inhibited by 58 per cent, lactic acid formation failed to occur in spite of the inhibited respiration. (Table VI). This makes it seem probable that the lactic acid formation which mature leucocytes, injured tissue, and tumor cells show in air and even in pure oxygen is not due to inhibited respiration but rather to a direct pathological change in their lactic acid fermentation ferment. That is to say, just as one could assume that the lactic acid formation in air caused by cyanide is due to a *reversible* blocking of the catalytic system of lactic acid fermentation, so that oxygen cannot react with it as long as the cell is poisoned, one could explain the metabolism type of tumor cells, injured body cells and mature leucocytes, as due to an *irreversible* change in the structure of this catalytic system, in such a way that its capacity of spontaneous reaction with oxygen has been lost once and for all.

After it had been established that oxygen tension has a decisive influence on the rate of respiration, *i.e.* on the sum of all oxidation processes which occur in the cell under normal conditions in a physiological medium, for instance in erythroblasts examined in blood plasma, it was interesting to see how this dependence of cellular respiration on oxygen tension might vary with variations

of the substrate in which oxidation takes place. We therefore measured the effect of oxygen tension on the oxidative deamination of amino acids in kidney cells (40-42) choosing this subject also because of its importance in clinical medicine, for it has been repeatedly stated, and with good reason, that in many diseases (in spastic, embolic and inflammatory processes of the kidney, in decompensated heart disease and hypertension, as well as in severe anemias) the kidney cells are poorly supplied with blood and hence with oxygen (62). Furthermore, the pathological conditions just mentioned were seen to lead frequently to renal insufficiency presenting the clinical picture of reversible uremic acidosis with increase of the urea concentration in the blood.

We have determined the deamination of *dl*-alanine, *dl*-valine, *dl*-leucine by measuring (6, 49) oxygen and amino acid consumption of kidney cell fragments and kidney slices of extreme thinness (0.05 mm.) in sodium chloride-bicarbonate-carbon dioxide and in sodium chloride-phosphate solutions with and without addition of amino acids. The oxygen consumption of the tissue slices not due to deamination of amino acids but to oxidation of keto acids formed in deamination etc., was inhibited by a preliminary one hour "shaking treatment" in the absence of oxygen at 38°C. The amino acid deaminase can stand this rough treatment; other oxidation ferments in kidney cells are more sensitive to it and perish after a short time. The oxygen tension was varied between 15 and 760 mm.Hg.

Below 60 mm.Hg oxygen, *i.e.,* just at those oxygen tensions actually occurring in the body under physiological and pathological conditions, we found the rate of deamination in surviving kidney cells very markedly dependent on variations of oxygen tension in phosphate solutions as well as in bicarbonate/CO_2. In intact tissue slices the rate of deamination of *dl*-alanine (0.05 M) was, at an oxygen tension of 41 mm.Hg, inhibited by 56 per cent compared to the deamination in air. The inhibition was completely reversible.

TABLE VII

Effect of oxygen tension of the oxidation of *dl*-alanine (M/20) in slices of kidney tissue. 37.8° C. Q_{O_2} = mm.[3] oxygen consumed in 1 hour by 1 mg. (dry weight) of kidney tissue. (42)

Volumes per cent of oxygen	Q_{O_2} Total oxygen consumption		Q_{O_2} Oxygen consumed in oxidation of *dl*-alanine
	I in NaCl phosphate without alanine	II in NaCl phosphate *dl*-alanine	II-I
20	6.4	18.5	12.1
5.4	4.5	9.9	5.4
20	5.4	17.9	12.5

The curve of the rate of deamination of *dl*-alanine, *dl*-valine, *dl*-leucine in kidney cells—resembling the oxygen saturation curve of hemoglobin at 38°C. (63)—showed a steep drop at oxygen tensions below 60 mm.Hg, but only a negligible change between 60 and 760 mm.Hg. This explains why Krebs in his extensive work on amino acid metabolism (64) has overlooked the important role of oxygen tension for the rate of deamination. In his experiments on the influence of oxygen tension on the deamination of *dl*-alanine in kidney extracts (65), he varied the oxygen tension only above 160 mm.Hg; the maximal difference he could find between 160 and 760 mm.Hg was an increase in the rate of deamination of only 11 per cent. At an oxygen tension of 25.8 mm.Hg we found in suspensions of kidney cell fragments the rate of deamination of *dl*-alanine (0.05 M) inhibited by 49 per cent, at an oxygen tension of 18.2 mm.Hg, the rate of demination of *dl*-valine (M/24) inhibited by 65 per cent as compared to

the rate of deamination in air. (Tables VII and VIII).

These experimental results seemed to provide an explanation for the phenomenon of reversible uremic acidosis in those states of kidney insufficiency in which the secretory renal functions are not impaired. They show that cessation of the chemical functions of the kidney is not necessarily due to anatomical destruction but can be caused by a temporary change in the gas concentration of the cell environment. Lowered oxygen tension in the kidney, no matter if caused by primary pathological processes in the kidney or by decompensated heart disease, hypertension, anemia, etc. reduces the renal amino acid deamination with corresponding ammonia formation to a fraction of the normal rate. The deamination of amino acids being the main source of urine ammonia (64), renal anoxia leads to disturbance of the acid-base equilibrium in the body; blood and tissue acids, reaching the kidney as sodium salts and

TABLE VIII

Effect of oxygen tension on the oxidation of *dl*-valine (M/20) in kidney cell residue. 37.8° C. Q_{O_2} = mm.[3] oxygen consumed in 1 hour by 1 mg. (dry weight) of kidney cell residue (42).

Volumes per cent oxygen	20		4		2.4	
	I —	II valine	III —	IV valine	V —	VI valine
Q_{O_2} Total oxygen consumption	2.86	14.28	2.56	7.70	2.27	6.20
Q_{O_2} Oxygen consumed in oxidation of *dl*-valine	II-I 11.42		IV-III 5.14		VI-V 3.93	

normally converted there into ammonium salts, are now, due to the inhibited ammonia production in the kidney, excreted as sodium salts into the urine; the blood bicarbonate drops and uremic acidosis ensues. But since this inhibition of deamination is a reversible reaction, the original rate of ammonia production is reestablished with the rise of the oxygen tension in the kidney to its original level, and the transient disturbance of the acid-base equilibrium disappears.

The finding of a reversible inhibition of deamination in the kidney by lowered oxygen tension may also offer an explanation of the mechanism of the reversible increase of blood urea found in cases of transient kidney insufficiency without secretory failure. Urea is the end product of deamination of amino acids in the liver, as is ammonia in the kidney. If the kidney is unable to carry out deamination, the portion of amino acids normally handled by the healthy kidney is offered to the liver and converted there into surplus urea; therefore the blood urea must rise, as a consequence of the reversible inhibition of the chemical functions of the kidney, even when its secretory functions are unimpaired and normal amounts of urea are excreted in the urine. Fig. 4 gives a schematic picture of the relation between the kidney and the liver with regard to the deamination of amino acids. It shows why a disturbance of the metabolism of the kidney cells must

lead to a drop of ammonia formation in the kidney and to rise of urea formation in the liver, and why, *vice versa*, a severe disturbance of the metabolism of the liver cells causes increased ammonia formation in the kidney, while the urea formation in the liver decreases (66, 67). If the disturbance of cell metabolism is of a reversible character, the pathological manifestations will disappear when normal conditions are reestablished. As soon as the kidney resumes its deaminizing activity, the vicarious plus-deamination in the liver ceases, and the urea formation is reduced to its original rate. States of transient uremia with acidosis as well as with high blood urea may thus be explained as being caused by the reversible inhibition of deamination of amino acids in the kidney cells under the conditions of lowered oxygen tension.

To the "fascinating simplicity of this conception," as he calls it, Meyer (68) raised the objection that though the experiments cited show that the rate of deamination of amino acids (ammonia formation) in kidney tissue decreased with decreasing oxygen tension, it was, however, very improbable that such conditions of low oxygen tension ever actually existed in the kidney. To support this experimentally, measurements of the oxygen tension of the urine of some patients were made, and since only slight differences were seen, the conclusion was drawn that *in vivo* the failure of the renal chemical function could not be explained as a result of lowered oxygen tension.

Schlayer showed, in reply to this objection (69), that it is impossible to set the oxygen tension of the urine on a par with the oxygen tension of the chemically active kidney tissue, since the oxygen tension of the urine is essentially determined by the oxygen tension of the extensive system of the collecting tubules and by that of the kidney pelvis, ureter, bladder and urethra, and moreover by Krogh's (70) "autoxidation" of the urine which, as Schlayer showed, changes considerably with the pH, being for instance 30 times higher in an alkaline than in an acid urine.

These discussions are quite instructive, because they indicate how the debate over the role of oxygen tension in biological oxidations has changed in the past years. Previously it was argued that cell respiration is entirely independent of oxygen tension and it makes no difference whether the oxygen tension in the cell milieu is high or low, for even at a minimal oxygen concentration the respiration remains at its maximal rate. Now, while the fact that the rate of cellular respiration is indeed markedly dependent on oxygen tension, is beginning to be accepted, the argument is raised from another angle by doubting the significance of this fact and contending that

NORMAL

LIVER · AMINO ACIDS · KIDNEYS

UREA · AMMONIA

KIDNEY INSUFFICIENCY

LIVER · AMINO ACID

UREA

LIVER INSUFFICIENCY

AMINO ACID · KIDNEYS

AMMONIA
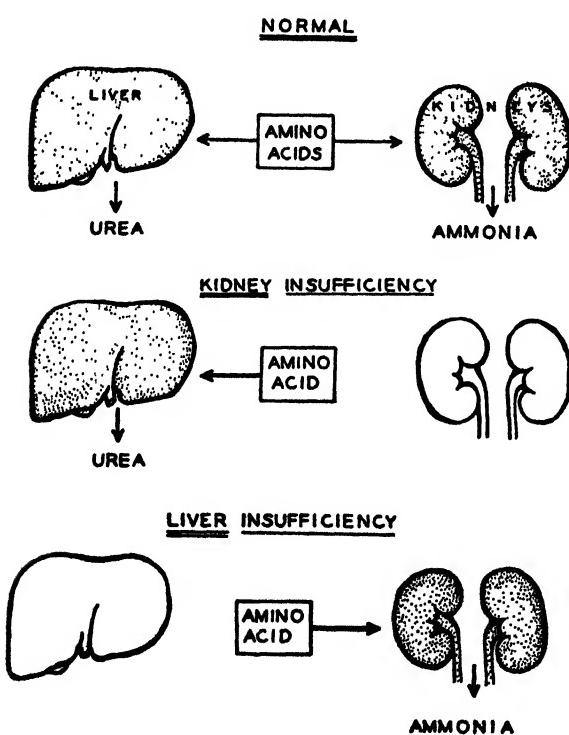
Figure 4.

such conditions of decreased oxygen tension never actually occur *in vivo*, although a series of exact measurements of oxygen tensions in the body have definitely shown the existence of "lows" in oxygen tension under various conditions (71-75).

I have chosen the instance of reversible uremia to point out the importance that must be attached to oxygen tension if one attempts to explain the mechanism of pathological conditions on the basis of biological laws. I do not want to go into other conclusions that could be drawn from this; perhaps at some future time we shall succeed in making therapeutic use of increased oxygen tension in the diseased tissue in such cases. Let me refer briefly to one other practical field where the same law of dependence of biological oxidations on oxygen tension suggests the application of just the reverse therapeutic measures: I mean the field of bacterial infection.

If, as our experiments have shown, bacterial respiration can be considerably suppressed by low oxygen tension, it could be anticipated that in bacteria which depend on respiration as their main source of energy, strangling this main source would bring about a state of minor chemical activity down to the point of *"vie latente"* (76) or "hibernation" (77). Indeed the growth of cultures of *Staphylococcus aureus* and of *Pneumococcus* that were examined in air and at lower oxygen tension under otherwise equal conditions, could be shown to be notably inhibited by decrease in oxygen tension (30, 38). But the reason why we fear the growth of bacteria in the body is, above all, that it increases the sum of their injurious effects, and while it is obvious that a smaller number of organisms will produce a smaller amount of metabolic products which in the case of pathogenic bacteria are harmful ("toxic") to the host, it follows from our experiments that also with an identical number of bacteria these "toxic" effects on the host will decrease with decreasing oxygen tension whenever the "toxic" substances are metabolic products formed in respiration. The effect of low oxygen tension on bacteria with mainly oxidative energy supply is thus seen to be a twofold one: a direct effect due to decrease of the chemical reactions, and, secondary to this, an indirect effect due to decrease of the bacterial growth. Both these effects of low oxygen tension are important for explaining the mechanism of numerous defense reactions of the body and may thereby be of value in deciding about the rationality or irrationality of a therapy to be applied. Formation of "closed off areas of inflammation" (75, 29, 77-81) and calcification may be the body's principle of producing low oxygen tension in a diseased organ in order to counteract injurious microorganisms. The same may be true for some forms of medical treatment. Pulmonary tuberculosis offers an interesting example (43, 45). The respiration of tubercle bacilli is, as we saw (Fig. 1), highly dependent on variation of oxygen tension, and respiration is virtually the only source of energy for the bacillus. If, then, practical experience has shown the curative effect of artificial pneumothorax, thoracoplasty, phrenic paralysis and high altitude, we feel justified in substituting for explanations like resting or relaxation of the lung, purer air, etc., the quantitative finding of the decreased energy supply of the tubercle bacillus due to the factor common to all these forms of treatment: the lowering of the oxygen tension in the lung. A treatment with high oxygen tension, therefore, though likely to give symptomatic relief in some instances, is certainly not advisable from the standpoint of the biology of the tubercle bacillus. The same applies for the high oxygen treatment in pneumococcus pneumonia, since pneumococci also thrive much better in high than in low oxygen tensions (30).
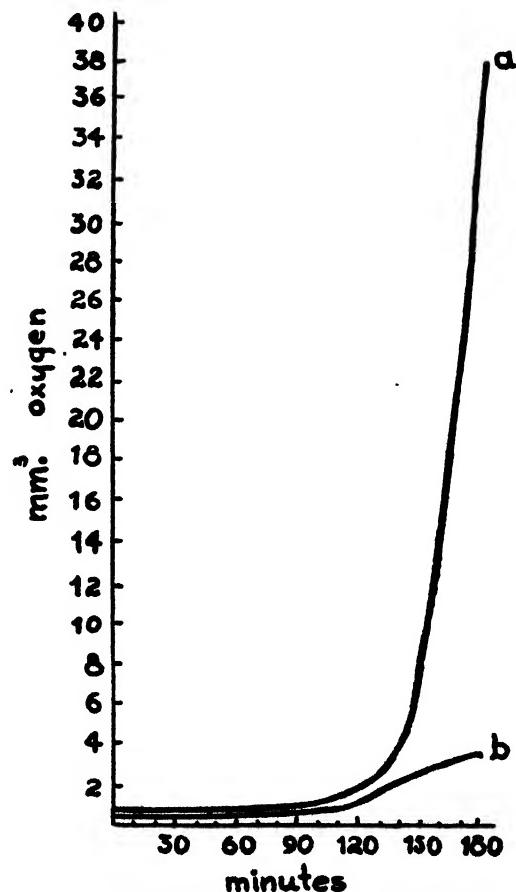
Fig. 5. Growth of pneumococci. Weight at beginning of experiment, 0.05 mg. (30). Curve a, 20 volumes per cent of oxygen. Curve b, 2 volumes per cent of oxygen.

But I do not want to enlarge any further here on practical applications and will briefly summarize the experimental results just reported.

1. We have to substitute for Warburg's all-or-nothing theory of respiration the law of the direct role of oxygen tension as a determining factor of the rate of respiration. This dependence of respiration on oxygen tension is found in cell-free plasma and in cells whose respiration is catalyzed by the iron-free yellow ferment as well as in cells whose respiration is catalyzed by the iron-containing phaeohaemin. It could be definitely proved that in these experiments on lowered oxygen tension, insufficient extra- and intracellular oxygen diffusion was not a limiting factor of respiration.

2. Together with the decrease of the rate of respiration there was also found in many cells a decrease of the respiratory quotient at lowered oxygen tension, revealing that the respiration was not only quantitatively but also qualitatively changed.

3. The sensitivity of respiration to oxygen tension is greatest in uninjured normal nucleated animal cells and young bacteria examined under physiological conditions, whereas old bacteria cultures, experimentally damaged mature leucocytes and non-nucleated red blood cells, even when their absolute rate of respiration was high, proved to be independent of variations of oxygen tension.

4. The oxygen affinity of the oxygen transferring ferment of cellular respiration is greatly influenced in many cells by the chemical composition of the suspension fluid, and in all cells by changes of temperature, just as is the affinity of oxygen to hemoglobin.

5. The Pasteur reaction, the disappearance or non-appearance of lactic acid formation in the presence of oxygen is not due to an effect of respiration on the lactic acid fermentation, but to a reaction of oxygen with the fermentation catalyst itself, no matter how the oxygen is transferred. There was no reciprocal relation between the oxygen affinity of the respiration ferment and that of the lactic acid fermentation ferment.

6. The effect of cyanide, carbon monoxide, ethyl cyanide, phenosafranine on cellular metabolism is explained as due to a direct reversible combination of these substances either with the catalytic system of lactic acid fermentation alone, or with catalysts of both systems, of that of respiration as well as that of lactic acid fermentation.

7. The lactic acid formation which mature leucocytes, injured tissue, and tumor cells show in air and in pure oxygen is explained as due not to their inhibited respiration, but rather to a direct irreversible change in their lactic acid fermentation ferment.

8. Just as cellular respiration, so oxidative deamination of amino acids in kidney tissue slices and kidney cell fragments is highly dependent on variations of oxygen tension.

9. The growth of bacteria whose main source of energy is respiration is diminished by lowered oxygen tension.

10. Decrease of oxygen tension in animal tissues might explain the incidence of pathological conditions, as that of reversible uremic acidosis, which one should attempt to counteract by high oxygen tension treatment. In contrast to this, diseases caused by bacteria whose main source of energy is respiration, should be treated by attempting to lower the oxygen tension in the affected organ.

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DISCUSSION

Dr. Stern: Kempner's experiments have brought out clearly the fact that it is the oxygen tension and not the respiration which is responsible for the Pasteur phenomenon. However, if I understood Kempner correctly, he has the concept that the Pasteur effect consists in the direct interaction of oxygen or hydrocyanic acid or carbon monoxide or any of the other agents cited with a component of the lactic acid fermentation system. Now if we remember that in the disturbance of the Pasteur effect lactic acid fermentation appears rather than disappears, then any combination of a component of the fermentation system itself with an inhibitor would, of course, tend to decrease rather than increase the fermentation.

I believe that Kempner's experiments can be interpreted as a de-inhibition of fermentation in the sense that carbon monoxide and hydrocyanic acid combine and thereby put out of action an agent which, under normal conditions, transfers oxygen to a component of the fermentation system thus decreasing the rate of breakdown of carbohydrate under aerobic conditions.

Dr. Kempner: The lactic acid fermentation system—let us call it x—reacts with O_2. This combination xO_2 is inactive, or as we generally express it, oxygen inhibits the lactic acid fermentation. In the absence of oxygen the lactic acid fermentation ferment x is active. It is also active in the presence of cyanide/air. This might be explained by assuming that, in analogy to the action of cyanide on the respiration ferment system, cyanide combines likewise with the lactic acid fermentation ferment system, pushing the oxygen away and thus activating the ferment.

$$xO_2 \text{ (inactive)} + \text{cyanide} =$$
$$x \text{ cyanide (active)} + O_2$$
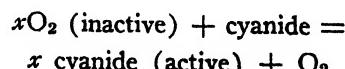
The essential result of the low oxygen tension and the cyanide experiments with regard to the Pasteur phenomenon is that the formation of lactic acid in the absence of oxygen or in the presence of cyanide is not the consequence of the decrease in respiration, but due to a direct effect of oxygen and of cyanide on the lactic acid fermentation system itself. O_2 affects x directly, regardless of how the oxygen is transferred to the fermentation system, whether by one of the catalysts which plays a role in respiration also or by some other oxygen carrier or "agent" in the cell.

Dr. Ball: I should like to ask Kempner if he does not think that in order to interpret his data in the way he has, he must not also make the assumption that under all the conditions which he has studied the energy requirements of the cell are not altered. Presumably the cell is utilizing

oxygen for the purpose of obtaining energy. What it does with that energy we know in part; how it does it we do not know. The cell carries on many functions and uses energy for many different purposes. Now, in the metabolism of a substrate we know that it may be broken down in stepwise fashion to CO_2 and H_2O and the energy in each step may perhaps be utilized differently. Therefore it seems to me possible that as the oxygen tension is lowered certain energy yielding reactions may stop before others do. Perhaps under such conditions oxygen would be utilized first for those reactions which are primarily essential for the cell. At any rate it seems a possibility that as the oxygen tension goes down, certain oxygen utilizing processes drop out, and that after you get down to a low oxygen tension where the cell cannot obtain enough energy through oxygen utilizing channels, you find the cell producing lactic acid and obtaining energy as in the anaerobic cycle.

I believe you indicated that only the oxidation of *d*-amino acids may be affected by lowered oxygen tension. I know of no evidence to indicate that *d*-amino acids are a normal constituent of the mammalian organism. The only evidence for the occurrence of a *d*-amino acid in living cells of which I know is that presented by Kögl and Erxleben for a *d*-glutamic acid in malignant tumors; I think we should therefore be a little careful in drawing too sweeping conclusions from experiments with this system.

Dr. Kempner: Before I go into the matter of the deamination of amino acids in kidney cells at low oxygen tensions, I will answer Ball's suggestion that under conditions of decreased oxygen tension, the cell stepwise discontinues the "less important" oxidation processes, only maintaining those oxidations which are of "primary importance" for cellular life, and that, if for these also the energy produced by oxidation becomes insufficient, the cell tries to obtain the necessary energy by the splitting of sugar to lactic acid. I am afraid that in this matter I am more pessimistic than Ball, for I do not think that if somebody has very little money at his disposal, he will spend that money only on essential things. So I am even more reluctant to agree entirely with the teleological interpretation that the cells by their "cleverness" 'should know what would be most reasonable for them to do under the conditions of lowered oxygen tension. The fact is that if the respiration rate is decreased to the same degree by different means, in one case—that of cyanide—lactic acid is formed, whereas in the other—that of low oxygen tension—lactic acid is not formed. That means that the two reactions, respiration and fermentation, are not coupled by the selective "cleverness" of cells trying to obtain their energy supply

one way or the other, but that these two reactions take place independently of each other, according to the different chemical constitution of the two ferments and the different reactivity of these with other substances, for instance with O_2. Both lactic acid fermentation and respiration are dependent on oxygen tension, but the oxygen affinity of the oxygen transferring ferment of respiration is different from the oxygen affinity of the lactic acid fermentation ferment just as, for example, the carbon monoxide affinity of the respiration ferment is different from the carbon monoxide affinity of hemoglobin.

That the rate of respiration drops with decreasing oxygen tension can be experimentally proved; how the energy requirements of the cell are affected by decreasing oxygen tension, whether they are identical with those at a normal oxygen tension or whether they too are decreased, is a question I would not venture to decide.

I do not know of any means of measuring the energy required by red blood cells, for example. The methods we have enable us to measure only the amount of energy produced by the cells. My experiments have shown that the energy produced by the erythroblasts decreases with decreasing oxygen tension. The experiments on the inhibition of bacterial growth under conditions of lowered oxygen tension may be of some significance here: they indicate that the energy produced at low oxygen tensions is not sufficient to meet the energy requirements for growth.

Dr. Barron: Kempner has given evidence of what I tried to show two years ago at the Symposium on anoxia in Baltimore, where I pointed out that the influence of the oxygen tension on the orientation of reactions was of great importance; so much so that metabolism of some of the oxidizable substrates could be altered considerably. At that time I pointed out, for instance, that pyruvic acid in the presence of oxygen oxidized to acetic acid and CO_2 in streptococcus, while in the absence of oxygen it went into acetic acid and formic acid. There is here a clear example of the orientation of reaction of pyruvic acid due to the oxygen tension. Of course we might come to the conclusion that the *degree* of orientation of pyruvic acid metabolism from the formation of acetic acid and CO_2 and the formation of acetic acid and formic acid will depend on the oxygen tension. At that time I also put forward the opinion that this orientation of reactions could be applied to the so-called Pasteur phenomenon.

Now I was very much interested to see Kempner's data on the influence of oxygen tension in respiration, R.Q. and lactic acid formation· When he diminished the oxygen content to 6.1 per cent he found an inhibition of respiration of something

like 60 per cent with no lactic acid formation, but at the same time he found the R.Q. greatly diminished.

We have here one more example of the influence of the oxygen tension on the orientation of reactions. In this case you are working with cells where the orientation of reactions has changed on lowering the oxygen tension in a manner such that no decarboxylative oxidations are produced, but only the dehydrogenations.

As Kempner, I think, realizes, his experiments on amino acid oxidations and the effect of oxygen tension on the rate of these enzymic reactions, may not apply to living cells.

We know perfectly well that d-amino acids are oxidized by activation and oxidation by alloxazins is dependent to some degree on the oxygen tension. It is perfectly correct to assume that in such a case the rate of oxidation will depend on the oxygen tension, but the normal cell does not oxidize d-amino acids. The oxidation of natural amino acids seems to go through the cytochrome system, and in such a case, of course, the influence of oxygen tension will be different from the influence of oxygen tension in the oxidation of d-amino acids.

Dr. Kempner: Barron has emphasized the fact, resulting from the experiments with erythroblasts, that variation of the oxygen tension not only changes the oxidation processes quantitatively, but also qualitatively, as indicated by the fact that the respiratory quotient of erythroblasts decreases with decreasing oxygen tension. I welcome this statement as a confirmation of what I said at the end of my paper: that the discussion about the role of oxygen tension in biological oxidations has changed considerably in the last three years. Formerly one followed the Warburg all-or-nothing theory, saying that variation of the oxygen tension does not change biological oxidations at all, and even at last year's Symposium on anoxia (Baltimore, 1938) one of the principal speakers, R. W. Gerard, stated that oxygen tension has in no way to be considered as a controlling factor in biological oxidations. Now there is no longer any doubt that biological oxidations are affected by variations of oxygen tension, and the main interest is already concentrated upon finding out the mechanism and the chemical end products of cellular respiration under conditions of lowered oxygen tension.

As to the question of "orientation": That changes of orientation in cellular metabolism occur if one alters the gas milieu from pure oxygen or air to complete anaerobiosis, has been a well known fact since the time of Pasteur's experiments. The first and most important discovery of such a change in orientation was the finding that yeast cells in oxygen or air break down the

sugar to water and carbon dioxide, and in the absence of oxygen to alcohol; or that the end product of the sugar metabolism of animal cells in air and in oxygen is water and carbon dioxide, and in the absence of oxygen, lactic acid. That even in the complete absence of oxygen the metabolic process can be changed by variations of the gas milieu, was described by F. Kubowitz with whom I worked on the effect of carbon monoxide on butyric acid fermentation (82, 83, 84). In argon the butyric acid bacteria metabolize the sugar to butyric acid, in carbon monoxide the butyric acid fermentation is inhibited by 92 per cent, the sugar breakdown only by 28 per cent. The end product of the sugar breakdown that persists in carbon monoxide is no longer butyric acid, but lactic acid. Barron's observation that streptococci metabolize pyruvic acid differently in the presence and in the absence of oxygen, is another significant example of such a change in orientation of the metabolic process.

The new observation in the experiments with erythroblasts is that here the change in orientation is not produced by decreasing the available amount of oxygen to zero nor by the inhibition of certain metabolic processes by carbon monoxide, but that such a change in orientation occurs in spite of the presence of a sufficient amount of oxygen merely through variations of oxygen tensions beginning at 5 vol. per cent, *i.e.* at tensions as they frequently exist under physiological conditions in the animal body.

As to the question of deamination of amino acids in kidney cells, I have worked with kidney cell extracts as well as with normal kidney tissue slices. I have measured the oxygen and the amino acid consumption of these slices not only in salt solutions with and without additions of dl-mixtures of alanine, valine, and leucine, but also in physiological plasma and serum of man, rat, and rabbit. It could be shown in this way that under conditions of lowered oxygen tension, kidney cells are actually inhibited in their rate of deamination, not only of the unnatural d-amino acids, but also of those naturally present in serum or plasma or in the tissue slices themselves.

I think I must emphasize again in this connection that the effect of lowered oxygen tension on cellular respiration was not only found in cell-free plasma and cells the respiration of which is catalyzed by iron-free yellow ferments, but likewise in cells the respiration of which is connected with the cytochrome system and is catalyzed by the iron-containing phaeohaemin.

Dr. Shorr: There are two points upon which I should like to comment. The first bears on Ball's suggestion that there may be other changes in the metabolism of the cell under low oxygen tensions to account for the failure of lactic acid

to appear. It is quite conceivable that the rate at which the chemical reactions of the cells go on under these conditions may be slower than at the higher oxygen tensions. Should this take place, the Pasteur mechanism might still be operating to prevent the appearance of lactic acid. This question could be examined experimentally by direct heat measurements. I wonder whether Kempner has calculated the lactic acid oxidative quotient of the bacteria under optimum conditions. Should the excess respiration over fermentation be very high, respiration might fall very considerably without lactic acid appearing.

The second point is in reference to the refreshing direction which the study of the role of bacteria in infection is taking. I refer to the recognition that chemical factors may play an important part, as against the purely immunological point of view. This demonstration of the effect of lowered oxygen tensions on bacterial metabolism is evidence of this character. The example of the tubercle bacillus is particularly interesting to me because of some work on this same organism reported by Loebel, Richardson and myself in 1933. We soon became aware of the lack of an adequate anaerobic mechanism in these bacteria to provide energy for survival and growth. They were poorly resistant to relatively short periods of anaerobic exposure. They were, however, remarkably resistant to a wide variety of other adverse conditions, including caseous material, provided oxygen were supplied. We therefore looked into the possibility that the tubercle might function as a limiting factor in the spread of this disease, by actually suffocating the tubercle bacilli which it contains. We could determine the respiratory metabolism of the monocytes which make up the tubercle, and calculate that a tubercle with a diameter exceeding 0.5 mm. should have an anaerobic center, unfavorable both for cells and for the tubercle bacillus. Cell death should occur with caseation, and bacterial growth inhibited. This general conception seems to be borne out by observation on the tubercle in disease. The bacilli are always found close to the rim of viable cells and not in the center of the tubercle—in other words as close as possible to the oxygen supply. When a tubercle ruptures, the bacteria are found in that portion of the caseous matter closest to the bronchus. That the tubercle actually does function in part to wall-off bacteria and cut off their oxygen supply would seem very likely from this evidence. Studies of bacterial infections along these lines should prove productive.

Dr. Kempner: The "lactic acid oxidative quotient" under optimal conditions can easily be calculated for red and white blood cells. At low oxygen tensions their respiration rate may fall very considerably without appearance or increased

formation of lactic acid, independent of the magnitude of the quotient: rate of respiration in air to rate of anaerobic lactic acid formation.

Calculating Warburg's so-called "excess of fermentation" for myeloblasts by substituting in the equation $U = Q_M^{N2} - 2Q_{O_2}$ the figures of Q_{O_2} in air and at 45 mm. Hg oxygen tension, given in Table VI, we find that U, negative in air, becomes positive with lowered oxygen tension:

$$U \text{ (air)} = 11.5 - 2 \times 7.7 = -3.9$$
$$U \text{ (45 mm. Hg O}_2\text{)} = 11.5 - 2 \times 3.2 = +5.1$$

Dr. Shaffer: It has always been to me a very fascinating view to take with respect to the respiration of polycellular organisms that the metabolism is dependent upon the rate of oxygen access. Whether you call that rate of oxygen supply or diffusion or oxygen tension locally I don't quite know. If one can suppose that under ordinary circumstances the rate of metabolism is really limited by the rate of oxygen availability, then it becomes reasonably possible to distribute the metabolism between fat, carbohydrates, and the derivatives of protein. If, however, you are going to imagine some sort of condition of a reciprocal yet dependent character that will fluctuate in proportion to the demand or relative participation of fat or carbohydrates into the total energy exchange, you are endowing our cells with a sort of omniscience which Ball seems disposed to attribute to them.

I would like to protest against the idea of there being any definable energy "requirement" of cells. It reminds me of the question, "How much does it cost to live in New York?". The answer, if you will remember, is "More than you've got!" I believe that principle is just as applicable with respect to total metabolism of cells as it is to living in New York. We know perfectly well that, although there is an approximate rough uniformity of the basal metabolism of certain human groups, if you go to different parts of the world or choose different age groups, or permit activity, with any one subject there is no such thing as "a requirement" of the individual.

A word or two in regard to possible conceptions of the mechanism of the relationship between lactic acid production and oxygen consumption. I have long held a certain concept, without, however, being able to get convincing evidence of its correctness. But I will give it to you for what it is worth. Let us remember that in all cells there are present both substrate and catalysts capable, in the absence of sufficient respiration, of producing lactic acid. (I am leaving out of account the oxidative removal of lactic acid once formed and taking only one side of the picture.) The problem is, how does respiration or oxygen consumption

keep the catalyst from its substrate, the result of which action is to produce lactic acid? Does the following experiment illustrate a possible mechanism?

One takes a solution of urease and puts it into a long glass tube with a porous membrane at each end, and some distance beyond has two platinum electrodes; put a current across that will electrolyze the urease and deposit the enzyme on one membrane. Then add a solution of urea, while keeping the enzyme polarized on the membrane. When the urease is piled up deposited on the membrane, there is a certain minimum rate of ammonia production by the action of the enzyme on the urea solution. One may measure it at intervals. So long as there is imposed a certain small potential to maintain the polarization of the urease against the membrane, you maintain a constant small rate of ammonia formation. Now remove the trickle charger, or short circuit the cell; the urease redissolves in the buffer solution with an enormous acceleration of urease activity and rate of ammonia formation.

If one imagines that the cell contains some sort of a device which, as a result of oxygen consumption, polarizes the enzyme on the surface away from the substrate—and that is easily conceivable, I think, although the mechanics are difficult to picture—so long as you polarize the membrane by respiration you keep the substrate and enzyme separate. Stop it and immediately, by diffusion, enzyme and substrate come together. This sort of thing, it seems to me, is what we can look for to account for the inhibition of fermentation by respiration.

Dr. Ball: The point I tried to make and apparently failed to do is this. A cell can apparently obtain energy by an anaerobic cycle in which lactic acid appears as the end product or by an aerobic cycle wherein CO_2 is the end product. Since lactic acid formation does not increase proportionately with lowered oxygen consumption, as you have shown, it would therefore appear possible that the energy consumption of the cell per unit of time is also altered by the conditions imposed upon it. Therefore any interpretation as to why lactic acid formation occurs, it would seem, must also consider this factor. Certain energy-furnishing reactions which utilize oxygen may proceed more readily under lowered oxygen tension than others. Lactic acid formation may or may not occur, depending upon whether these reactions are capable of meeting the energy demands made by the cell per unit of time. Such a situation appears to occur in muscle where lactic acid accumulation may occur if the sudden demand for energy is greater than that which can be supplied by oxygen utilizing processes.

Dr. Shaffer: The polarization of the cell, keeping the substrate away from the enzyme, might be accomplished at lower than the usual oxygen consumption. It is not necessary that the rate of lactic acid production begin immediately to increase as soon as the rate of oxygen consumption begins to decline.

Dr. Barron: When you work in 6 per cent oxygen you find a lowered respiration, and no influence on lactic acid formation. But you also have no CO_2 formation. Isn't that so?

Dr. Kempner: That is certainly true with the CO_2 formation of erythroblasts; in other cells, for instance in tubercle bacilli, the R.Q. remains constant.

Dr. Barron: Now in that case, if I say that the lactic acid formed at 6 per cent oxygen was oxidized to pyruvic acid, you will have the oxidation of lactic acid without CO_2 production. As Ball has pointed out, the influence of oxygen tension on the individual rates of oxidation may be different. If lactic acid oxidation to pyruvic acid goes through the cytochrome system, the influence of the oxygen tension on this oxidation will be less. As a consequence the small amount of lactic acid produced at 6 per cent will be oxidized to pyruvic acid. So I still maintain that you have lactic acid production at this oxygen tension, but this lactic acid might have been oxidized to pyruvic acid. I would say that lower oxygen tension acts differently on different enzymic reactions, and that the influence of oxygen tension on dehydrogenations is less than the influence of oxygen tension on oxidations in decarboxylative oxidations. Oxidations with no CO_2 formation are produced as a consequence; at a tension of 6.1 per cent you will have diminished oxygen production, but the rate of oxidation of lactic acid to pyruvic acid still persists. As a consequence you find no lactic acid, and as a consequence the CO_2 formation goes down; your diminished CO_2 formation may thus be explained.

Dr. Barker: It is known that carbohydrate oxidation can take place in intact tissues under the influence of iodoacetic acid, although the formation of lactic acid and of pyruvic acid is inhibited. If one assumes, on the basis of this evidence, that there is no dependence of respiration on a preliminary anaerobic phase, there is no apparent *a priori* reason for expecting either lactic or pyruvic acid as the oxygen tension is lowered.

Dr. Kempner: It was interesting to hear Barron's hypothesis about the mechanism of the metabolism change of erythroblasts at low oxygen tension. His assumption that the low respiratory quotient might be explained by their oxidizing some lactic acid, formed intermediately, to pyruvic acid is, however, experimentally disproved. For, considering the high respiratory rate of erythroblasts, even if only 40 per cent of this respiration remained, the pyruvic acid would be present in

large amounts and would easily show up in the manometrical experiment, where, as Warren has pointed out, every acid formation is detected by the carbon dioxide liberated from the bicarbonate of the suspension fluid.

Dr. Stern: In order to clarify our ideas about your concept about the Pasteur effect, would you put your scheme on the blackboard? I think we still disagree about this.

Dr. Kempner: I think any scheme on the mechanism of the Pasteur effect which one could put on the blackboard at present would be mere speculation unsupported by experimental facts. Warburg used to make a differentiation between two kinds of chemistry, experimental chemistry and so-called paper chemistry. Limiting ourselves to the former, all we can say up to now is that the Pasteur phenomenon, which means the disappearance of lactic acid in the presence of oxygen, is not due to a decreased rate of respiration, but to a direct action of the oxygen on a component of the lactic acid fermentation system.

Dr. Stern: Now in the old concept no separate Pasteur agent is assumed, but the effect of oxygen is pictured as an effect through respiration on fermentation.

Kempner believes that oxygen acts directly on a component of the fermentation system. His evidence that respiration has nothing to do with the effect I consider as unequivocal and supported not only by Kempner's experiments but by several other workers in the field. The question resolves itself then: do we have to assume the existence of a separate Pasteur agent?

To my first remark Kempner replied that in his concept oxygen combines with a component x contained within the fermentation system, thereby producing a reversible inhibition of this component. Upon adding carbon monoxide or HCN the oxygen is displaced from the enzyme and glycolysis is permitted to occur. However, this simple concept meets with a great difficulty in that it implies that these substances which compete with the oxygen for this component in the fermentation system form a complex with this x which is catalytically active. Except for certain experiments on the effect of cyanide on the oxidation of unsaturated fatty acids by hematin there have been no cases reported where the combination of carbon monoxide or cyanide with a catalyst has preserved the catalytic activity. Therefore I feel that we have to assume the existence of a Pasteur agent which normally (*i.e.* in oxygen) inhibits a component of the glycolytic system. However, when it is poisoned by carbon monoxide or by cyanide the inhibition is removed and the activity of the glycolytic system restored.

THE METABOLISM OF DAMAGED CELLS AND TISSUES

WALTER FLEISCHMANN

The metabolism of cells and tissues after exposure to injuries has been studied by various authors, but as these studies have been made from different points of view and often without reference to each other, it seems justifiable to try to review them.

The starting point of the problem is Warburg's observation (1) that cells of benign and, to an even greater degree, of malignant tumors differ fundamentally from normal cells in their energy-supplying metabolic reactions. In the metabolism of cancer cells the respiration-glycolysis ratio is disturbed and a great amount of lactic acid is formed under aerobic conditions. This is because the rate of glycolysis is high as compared to the rate of respiration. In normal undamaged cells with some exceptions, such as the retina, the oxidative metabolism is far greater than the glycolytic metabolism and under aerobic conditions no lactic acid appears. It was somewhat disturbing and not in accordance with these conceptions when Bakker (2) reported that exudate leucocytes from the rabbit have a small rate of respiration, together with high aerobic glycolysis. According to Bakker, leucocytes conform to the type of metabolism considered characteristic for the cancer cell. The problem arose: Have leucocytes the metabolism of cancer cells? I had the good fortune to enter Warburg's laboratory at that time. As I had had some experience in the isolation of leucocytes, Warburg suggested that I repeat Bakker's work. Although we (Fleischmann and Kubowitz, 3) could not confirm the very low respiration of leucocytes reported by Bakker, we found that exudate leucocytes from rabbits showed a type of metabolism much more closely resembling that of tumor cells than did other normal cells. We used the same methods as Warburg had used in his studies on tumor cells. Our measurements have been confirmed recently by MacLeod and Rhoads (4).

Our next step was to examine white blood cells isolated from blood, through centrifuging. We used citrated blood of geese, from which the leucocytes can be isolated very easily. These cells showed aerobic glycolysis to a much lesser degree than the exudate leucocytes, and we came to the conclusion that aerobic glycolysis was not a proof of tumor-like nature of the white blood cell, but a sign of ageing of the white blood cell in the blood stream, and finally of damage of the cell under the conditions prevailing in the exudate. Respiration was evidently more sensitive to injury than was glycolysis. In the process of ageing, or through damage, oxidation decreased so far as to become insufficient for oxidizing the total amount of lactic acid, the result of the relatively unimpaired glycolytic system. A series of measurements by Fujita (5), who continued our work in Warburg's laboratory, showed this very clearly. He examined the metabolism of bone marrow cells of the rat in Ringer's solution and found the following values (Fig. 1).
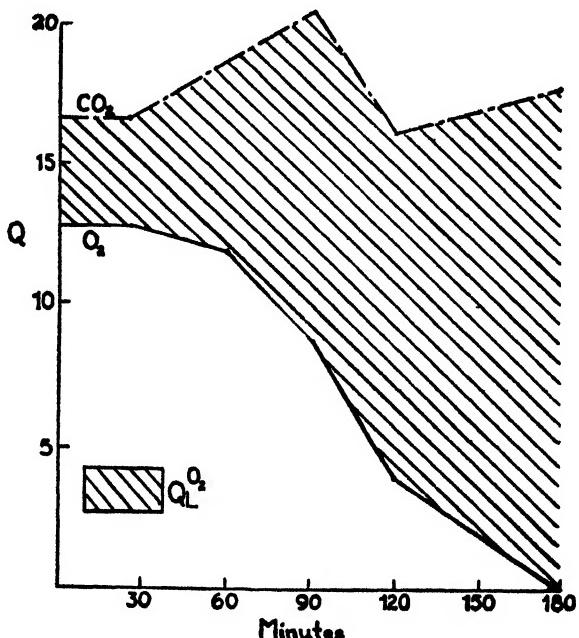
FIGURE 1

	Q_{O_2}	$Q_L^{O_2}$	$Q_L^{N_2}$
First half hour	—12.9	3.8	25.8
Second half hour	—11.9	6.8	25.4
Third half hour	— 8.8	11.6	21.6
Fourth half hour	— 3.7	12.4	20.8
Fifth half hour	— 2.0	14.7	20.6
Sixth half hour	0	17.5	18.9

	Q_{O_2}	$Q_L^{O_2}$	$Q_L^{N_2}$	Reference
Rat (liver)	—12	0	3	(1)
Rat sarcoma	— 9	16	34	(1)
Blood leucocyte (goose)	— 5	2	12	(3)
Exudate leucocyte (rabbit)	— 4	14	23	(3)
Exudate leucocyte (rabbit)	— 4.6	17	25	(4)

The aerobic glycolysis of leucocytes seemed important in connection with two problems, the problem of the nature of the leucemic cell and the problem of inflammation. Since both lines of research have furnished material bearing on our problem, I shall discuss them briefly.

The question whether the metabolism of leucemic cells is the same as that of cancer cells is important for the theory of leucemia. Some consider this disease as a type of tumor formation, others as an excessive production of essentially normal young cells. The work in this field has recently been reviewed by Kempner (6) and so I shall give you only the findings bearing immediately on our subject. Peschel (7) examined blood cells from patients with lymphatic leucemia, determining oxygen consumption, and aerobic lactic acid formation by the Warburg technique. Peschel showed that under strictly physiological conditions the immature leucemic lymphocytes have a purely oxidative metabolism and do not show any formation of lactic acid under aerobic conditions. Peschel concluded from his observations that, since mature and immature lymphocytes have a very different metabolism in that the immature leucemic lymphocytes conform to the pattern of the purely oxidative metabolism of normal cells, it is proven that leucemic lymphocytes with respect to their metabolism are not tumor cells but normal young tissue cells. Furthermore, the aerobic glycolysis of mature lymphocytes is only a manifestation of their dying off in the blood or exudate. Schlossmann (8), using material and technique very similar to Peschel's, came to the same conclusions. Kempner (6) has studied the metabolism of blood cells from a great number of cases both of lymphatic and of myelogenous leucemia. His principal conclusion is that lymphoblasts, as well as myeloblasts, have a purely oxidative metabolism and do not form lactic acid under aerobic conditions. Aerobic glycolysis which occurs in more mature leucocytes and lymphocytes was found to be a symptom of their ageing and dying off. Myeloblasts and lymphoblasts exhibit the metabolism of uninjured normal cells, whereas leucocytes and lymphocytes show the metabolism of injured cells. These observations seem to show definitely that our hypothesis is correct. The ageing of a cell in the blood stream or its wandering out of the blood into an exudate results in an aerobic glycolysis.

	Q_{O_2}	$Q_L^{O_2}$	$Q_L^{N_2}$	Reference
Lymphoblast	−6	0	11	(6)
Myeloblast	−8	0	12	(6)

Is this aerobic glycolysis of leucocytes a phenomenon which can only be observed *in vitro* or

does it occur in the body? Kempner and Peschel (9) examined mononuclear and polynuclear cells obtained from sterile blisters of the skin. The type of metabolism was exactly the same as in the exudate leucocytes of animals, namely, high glycolysis, relatively low respiration and lactic acid formation under aerobic conditions. The absolute figures were considerably higher than those reported in the animal experiments, but the ratio of aerobic glycolysis to respiration was of the same order of magnitude. That this type of metabolism not only occurs *in vitro* after removing the cells into the closed chamber of the manometer, but also in the inflamed area, was proven by the fact that products of leucocyte metabolism were found in the fluid filling these blisters. The sodium lactate concentration was above 125 mg. per cent as against 10 mg. per cent in normal tissue fluid; the bicarbonate had decreased to half the usual amount and the pH had dropped to values as low as 6.2. The sugar concentration of sterile blisters of the skin was zero, against 100 mg. per cent in normal tissue fluid. That these chemical changes are due, for the most part, to the aerobic glycolysis of the leucocytes is further shown by the observations of Barnett and MacKenney (10), who compared the lactic acid content of exudates and transudates with the lactic acid content of the blood in a number of patients. While in transudates the lactic acid level is the same or less than in the blood, in exudates it is increased up to 2400 per cent of the lactic acid level of the blood.

Aerobic glycolysis of the mature white blood cell may play an important role in inflammation. It has been stressed by several workers in this field that the faculty of the leucocyte to use glycolysis as a source of energy must be of great importance when the leucocyte has to function under conditions of very low oxygen tension. In 1927 I spoke of the leucocyte as a facultative aerobe (Fleischmann, 11). Barron and Harrop (12) came to the conclusion that "glycolytic activity seems to be a more stable type of activity and its greater magnitude in the polynuclear leucocyte must be vital to the activities of this cell when it is placed, as must often be the case, in conditions of very low oxygen tension."

This is especially true for one important function of the leucocyte, namely phagocytosis. Phagocytosis depends on glycolysis, not on cell respiration. A leucocyte deprived of the access to free oxygen, will take up bacteria or solid particles only if the medium contains enough glucose to maintain glycolysis (Fleischmann, 11). Poisoning of leucocytes with sufficient cyanide to inhibit oxygen uptake does not affect phagocytosis, but phagocytosis is inhibited if both respiration and glycolysis are poisoned by sodium fluoride. These experiments in Durig's laboratory were confirmed

and amplified by Höber and Ferrari (13). These authors showed that mono-iodo-acetic acid in concentrations of 1:10,000 to 1:30,000 inhibits phagocytosis. This is a concentration which inhibits glycolysis specifically. On the other hand concentrations of cyanide which inhibit cell respiration but do not affect glycolysis have no inhibiting effect on phagocytosis. Experiments on the storing of dyes through the Kupffer cells in the perfused isolated liver showed the same thing. Phagocytic activity is not inhibited by cyanide but by mono-iodo-acetic acid. Höber comes to the same conclusion as we did, namely that in phagocytosis glycolysis is the energy-yielding reaction. The importance of this fact in suppurative inflammation is evident. In the earlier stages of inflammation, when the oxygen supply is low but the glucose supply is still adequate, the glycolytic system, being more resistant than the respiratory system, warrants this important function of the leucocyte.

What happens to the metabolism of leucocytes under the conditions of advanced inflammation? We know that often, for instance in the case of an abscess, the inflamed area is separated by a barrier from normal tissues. Ruth Lohmann (13) has examined the influence of low concentrations of bicarbonate and of glucose on tissue metabolism and has found that glycolysis is more sensitive to this type of damage than respiration. When the number of leucocytes is great it may happen that within a short time so much bicarbonate and glucose may be used up in aerobic glycolysis and respiration that the aerobic glycolysis is reduced to a fraction of its optimal rate or even disappears. Under extreme conditions of inflammation, a purely oxidative metabolism may result, but this must be considered as a terminal stage in the life of the exudate leucocytes, for under these conditions glucose as a source of energy is lacking. These are conditions which are most unfavorable for all bacteria which depend on oxygen and sugar as sources of energy. They are severely damaged or even starved to death. Aerobic glycolysis thus plays an important part in preparing unfavorable conditions for the pathogenic bacteria. An interesting idea has been brought forward recently by Kempner (5). Since the myeloblastic cells lack aerobic glycolysis completely, they are unable to produce the conditions unfavorable for pathogenic bacteria. The function of mature leucocytes cannot be taken over by immature leucemic cells. This must lead to a serious danger to the body in cases of leucemia. Not the presence of myeloblasts or lymphoblasts but the absence of mature white blood cells is responsible for the weakened resistance to infections in patients suffering from leucemia.

Is the change in metabolism from a purely oxi-

dative type to aerobic glycolysis as a result of injury confined to the white blood cell? A few examples may show us that these changes, first studied in the white blood cell, are only a special case of a general principle.

Walthard (15) has shown that in fatty degeneration of the liver the same change takes place.

	Q_{O_2}	$Q_L^{O_2}$
Liver of normal mouse	−12.3	0.6
Fatty degenerated liver	− 4.7	2.8

Similar changes take place in the testis of the rat after hypophysectomy. Reiss, Druckrey and Hochwald (16) have shown that respiration is decreased and aerobic glycolysis increased in the atrophied testes of rodents after hypophysectomy. The atrophy of the testes was verified by histological sections.

	Q_{O_2}	$Q_L^{O_2}$	$Q_L^{N_2}$
Testis of normal rat	−10.7	2.0	9.6
Testis of rat after hypophysectomy	−5.9	4.0	7.2

Another type of damage is that due to irradiation with X-rays. Loew-Beer and Reiss (17) irradiated Jensen-sarcoma in rats *in vivo*, then excised the tumors and measured their metabolism *in vitro* by Warburg's technique. Again the effect on respiration is much more marked than that on glycolysis.

	Q_{O_2}	$Q_L^{O_2}$	$Q_L^{N_2}$
Jensen sarcoma, non-irradiated	−8.5	26.1	31.8
Jensen sarcoma, after irradiation	−1.6	22.7	32.3

This effect of irradiation has been confirmed by various workers. Kahlstorf (18) has shown that irradiation which hardly affects the metabolism of the liver brings about a marked decrease in respiration and increase in aerobic and anaerobic glycolysis in the testes. This illustrates the fact that germinative tissues are especially sensitive to irradiation. The tissues were irradiated with 600 r in the body, then quickly removed and examined by the Warburg technique.

	Q_{O_2}	$Q_L^{O_2}$	$Q_L^{N_2}$
Liver, normal	−10.9	0.7	1.8
Liver, irradiated	−10.5	0.7	2.4
Testis, normal	−10.7	1.4	8.9
Testis, irradiated	−8.2	2.9	12.5

Fleischmann and Laszlo (19) have studied the influence of irradiation of avian blood cells by radium emanation. Blood cells of the goose suspended in Ringer's solution containing radon (5 millicurie per 1000 cc.) take up only 75 per cent as much oxygen as is taken up by controls suspended in radon-free Ringer's solution (Fig. 2).
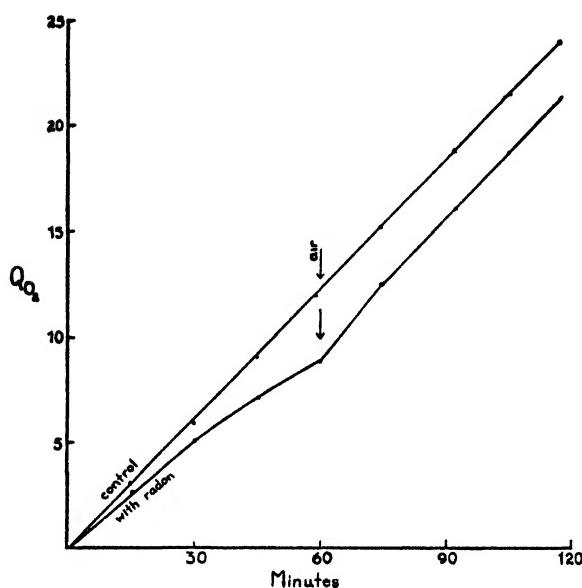
FIGURE 2

	Q_{O_2}
Erythrocytes (nucleated), control	—12.0
" , with radon	— 9.1
" , after removal of radon	—12.2

After removing the radium emanation by a stream of air the rate of oxygen uptake returns to normal and continues for several hours at the same rate as the oxygen uptake of the control cells. The controls were submitted to the same procedures as the irradiated cells (as, for instance, the blowing of air through the respiration vessel of the Warburg apparatus). These experiments indicate that the inhibition of respiration due to irradiation with radon is completely reversible. The cells show no morphological signs of injury with this type of irradiation, which is therefore to be considered rather as a reversible inhibition of the enzyme system than as a definite injury. The nuclei of the irradiated red blood cells were not stained with neutral red, a sign that the cell is not injured. The living nucleus is unstainable, and we and others have often found the taking up of neutral red by the nucleus to be the first morphological sign of cell injury.

Isolated cells, such as avian erythrocytes or leucocytes, are especially suitable objects for studies on the effects of injury. Removing them from the body does not in itself involve such serious injuries as, for instance, the examination of slices of liver or other parenchymatous organs.

The carbohydrate metabolism of tumor cells has been studied very thoroughly by Crabtree (20). He confirmed the observation that the two energy-yielding processes of respiration and glycolysis are not equally sensitive to radiation. A selective diminution of respiration occurs as an effect of radiation, while aerobic glycolysis remains relatively unimpaired. "The vital and labile respiratory system of mammalian tissue is more vulnerable to radiation than the glycolytic system and plays a dominant part in the biological response of irradiated tumor cells. "Fixing" the respiration of tumor tissue with cyanide at 37.5° makes it more sensitive to radiation. Irradiation of tumor tissue at body temperature either under aerobic or under anaerobic conditions or in the presence of HCN causes a selective lowering of respiration whilst glycolysis remains relatively unaffected." Respiration is primarily damaged at this temperature. Irradiation of tumor tissue at low temperature under either of the conditions mentioned causes a selective lowering of glycolysis, while respiration is hardly affected. Irradiation of normal tissues at normal temperatures does not have this effect. All of these experiments were carried out by irradiation of the tissues in vitro. With the exception of irradiation at low temperatures, which is, after all, an unphysiological condition, the effect of injury on tissue metabolism follows the same pattern after various kinds of injury. Oxidations are more sensitive than glycolysis and the appearance or the increase of aerobic glycolysis is the result.

Most of the earlier workers in this field compared the metabolism of damaged tissue with that of undamaged tissue, but failed to study the changes of metabolism with respect to the time factor. Some newer observations by Druckrey (21) and by Kaunitz and Selzer (22) shed some light on the changes in metabolism after injury. Druckrey observed that sudden injury to a cell or tissue is followed by an increase of respiration. This is shown by a graph from Druckrey's paper on the influence of quinine on tissue respiration (Fig. 3). The metabolism of slices of liver was measured by the Warburg technique and at a certain point quinine was added without opening the closed system. An enormous increase in oxygen uptake resulted; however, the increase in glycolysis was even greater and the result was the appearance of aerobic glycolysis. The same type of curve results if the tissues are exposed to low oxygen pressure or are injured mechanically.

The experimental conditions are very complicated when studying the tissues of warm blooded
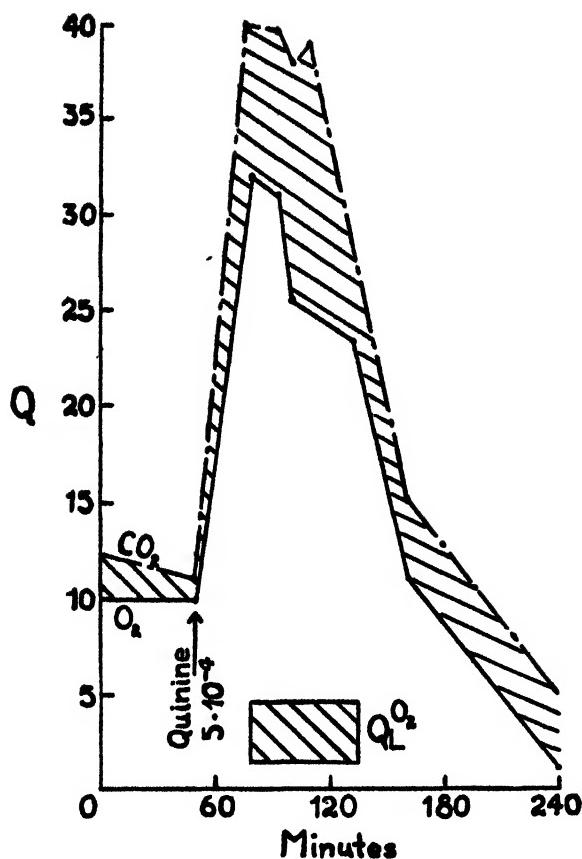
FIGURE 3

animals, and it seemed necessary to look towards other objects for studying the mechanism of damaged cells. Since the pioneer work of Warburg (23), we know that fertilization in the sea urchin egg is followed by an increase in oxidation. Runnstroem (24), who has made very careful studies in this field, has shown that after fertilization aerobic acid formation takes place.

Time after fertilization	X_{O_2}	$X_L^{O_2}$	$-\dfrac{X_L^{O_2}}{X_{O_2}}$
0 to 10 minutes	−6	14.2	2.37
10 to 20 minutes	−8.2	7.46	0.92
20 to 30 minutes	−9.4	6	0.64
30 to 40 minutes	−12.8	5.8	0.54

Druckrey, Brock and Herken (25) have studied the response of oxygen uptake to injury in fertilized and unfertilized sea urchin eggs. This response is unspecific. A sharp rise after injury is followed by a decline to subnormal values. Acids, bases, hypo- and hypertonic solutions, quinine, and other chemical agents injurious to the cell produce

the same type of reaction. Not only was the type of reaction the same after injury by one of these different agents, but it was the same in fertilized and in unfertilized eggs. A careful study of the changes in oxygen consumption of sea urchin eggs showed that the sudden rise of oxidation after fertilization is in some cases followed by a decline and only after this decline does the gradual increase of oxidation set in.

The close resemblance of the changes in metabolism after injury and after fertilization, and the fact that many agents which damage the cells lead to artificial activation, indicate a common cause of these reactions in metabolism. Druckrey and his co-workers see this common cause in a change of permeability of the cell membrane.

It is known that fertilization and artificial activation are accompanied by changes in permeability. This has been shown by changes in electrical conductivity and by other methods. Lillie (26) regards the essential element in the artificial segmentation under the influence of certain salts as an increase in the permeability of the cell membrane. Moreover, all substances which act as activators are known to influence permeability; this is especially true for the narcotics. On the other hand it is known that injury can lead to a change in permeability of the cell membrane. Lack of oxygen, for instance, is followed by an increased permeability of the muscle membrane, as has been shown by Cl estimations (Winterstein and Hirschberg, 27).

It is quite in accordance with current views to consider the changes in metabolism after fertilization and after injury as consequences of changes in permeability of the cell membrane. Both fertilization and injury are accompanied by changes in permeability and, furthermore, all substances which can act as artificial activators have a cytolytic property. They can therefore produce a process resembling fertilization and an injury fatal to the cell.

The close connection between fertilization, changes in cell surface, and oxidations is made clear through studies on the temperature coefficient of the fertilized sea urchin egg by Gerard and Rubinstein (28). Activation, according to these authors, depends in part on surface changes. The activator thus tends to disorganize existing cell surfaces and so to liberate for free reaction in solution the partly bound or inactive enzyme or substrate. Complete cytolysis as the extreme change in permeability would act in the same way. Both on fertilization and on cytolysis the temperature coefficient falls to half the resting value.

Kaunitz and Selzer (22) have examined the tissue metabolism of animals in which the conditions of serous inflammation have been produced

experimentally. Serous inflammation is defined by the school of Eppinger as "the entity of all tissue damages due to disturbance of biological permeability" (Kaunitz). Serous inflammation of the parenchymatous organs can be experimentally produced by poisons such as allyl-formiate. Rats in which this condition has been experimentally produced were killed and slices of their livers placed immediately in the chamber of the Warburg manometer. These tissue slices invariably showed an increase in oxygen consumption, which dropped only after reaching a peak at the end of the first hour. The tissues of control animals never showed this increase. In serous inflammation the changes in permeability of the cell membranes have been demonstrated by electrolyte determinations and by vital microscopy. It could be shown that certain dyes, like fluorescin, are taken up out of the blood stream by tissues of animals poisoned by allyl-formiate. In normal animals fluorescin was not taken up by the tissues. Independently from Druckrey and starting from clinical observations, Kaunitz came to the same results and drew the same conclusion: "The increased oxygen uptake of damaged tissues is due to a change in permeability."

The fact that aerobic glycolysis appears in damaged tissues at the same time as artificial respiration, is rather surprising and seems at first difficult to reconcile with the current views on the Pasteur effect. We expect aerobic glycolysis to appear if respiration is decreased and not if it is increased. I shall not try to discuss this problem fully but will only quote an explanation which Dixon and Holmes (29) have offered, which at the same time explains the sparing action of oxygen on glycolysis of normal cells. Oxygen, according to these authors, affects cell permeability so as to set a limit to the rate at which glucose can reach the cell enzymes. Inhibition of the Pasteur effect consists of a removal of this limitation, so enabling more glucose to reach the intracellular enzymes. This increased permeability results in an increased rate of both respiration and lactic acid formation. The view that agents which inhibit the Pasteur effect do so by affecting cell permeability is readily acceptable in the case of potassium. Dixon and Holmes found that in the presence of high concentrations of potassium salts both the lactic acid production and the rate of respiration of slices of brain are greatly increased. Potassium ions may affect cell surfaces by converting a water-in-oil emulsion to an oil-in-water emulsion. It is in accordance with these views that the inhibition of the Pasteur effect brought about by potassium is reversed by calcium and not by sodium. A similar explanation is brought forward to explain the changes in cell metabolism due to lack of oxygen. Here again a change in permea-

bility of the cell membranes is the principal factor involved. If animals are kept for some time under low oxygen tension, the organs show signs of serous inflammation. If slices of liver of such animals are placed in the chamber of the Warburg manometer immediately after killing they show an increasing oxygen consumption quite similar to that of the tissue of animals poisoned with allyl-formiate (Kaunitz and Selzer). This increase in oxygen consumption during the first hour after removal from the body *in vitro* is in the first instance due to changes in permeability. A second factor is possibly that an oxygen debt has been acquired during asphyxia. Brock (30) states that if tissues are sensitive to lack of oxygen and are kept under anaerobic conditions, they react with increased respiration if they are brought back again into aerobic conditions. Brock comes to the same conclusions as Kaunitz and Selzer: oxygen debt and changes in permeability are the causes of this rise in oxygen consumption. It seems possible that excessive oxygen consumption occurs in isolated tissues just as in whole animals as a result of an oxygen debt.

These effects of asphyxia on the rate of oxygen supply give an explanation of the convulsions due to anoxemia of the brain (Dixon 31). If the rate of oxygen supply to the brain is diminished and approximately anaerobic conditions are realized in some of the brain cells, the rate of consumption of glucose by the cerebral cortex is greatly increased. Stimulation of metabolism may be accompanied by stimulation of the nerve centers and thus convulsions are really due to intracerebral anoxemia. A still further increase may lead to capillary collapse, and so impede cerebral circulation as to deprive the brain cells of glucose. The rate of cerebral metabolism is now substantially reduced, even below its normal level, and irritation thus gives way to paralysis. Stone (32) has shown that the lactic acid content of brain is increased by cyanide convulsions and suggests that convulsions may be associated with increased tissue activity. Dixon finds the amount of glucose utilized by rabbit cortex increased by 250 per cent by addition of 2×10^{-3} molar sodium cyanide. While respiration is diminished by 50 per cent, aerobic lactic acid production is greatly stimulated. Increased nervous activity is connected with consumption of glucose. These facts show clearly that the reactions in cell metabolism to injuries of various kinds occur not only *in vitro* or under experimental conditions, but that they occur in the body under pathological conditions.

Not only pathological conditions bring about this change in tissue metabolism. We have seen that fertilization of the sea urchin egg is followed by the same change. Brock, Druckrey and Herken (33) have shown recently that stimulation of

the submaxillary gland *in vitro* is followed by the same type of change in metabolism as injury. The experiments were carried out in isolated salivary glands of rats *in vitro*, acetylcholine being used as stimulant. If the effect of acetylcholine is inhibited by atropine the change in metabolism does not take place. It is remarkable how much the metabolism curves after physiological stimulation (Fig. 4) and after damage through quinine, for instance, (Fig. 5) resemble each other. In both cases the change in metabolism is reversible. Metabolism returns to normal and the experiment can be repeated with the same result after some time. It is to be assumed that changes in permeability due to stimulation are the causes of the rise in oxygen uptake.

The observations on the influence of injury on the energy-yielding metabolic processes of the cell can be summarized briefly, as the pattern in the change in metabolism is strikingly uniform.

(1) Respiration is more sensitive to injury than glycolysis. In gradual ageing or degeneration of cells respiration decreases more rapidly than glycolysis, and aerobic glycolysis appears or is increased.

(2) In many instances injury is followed by an increase in respiration. The rate of respiration rises to a peak and then declines. Contrary to
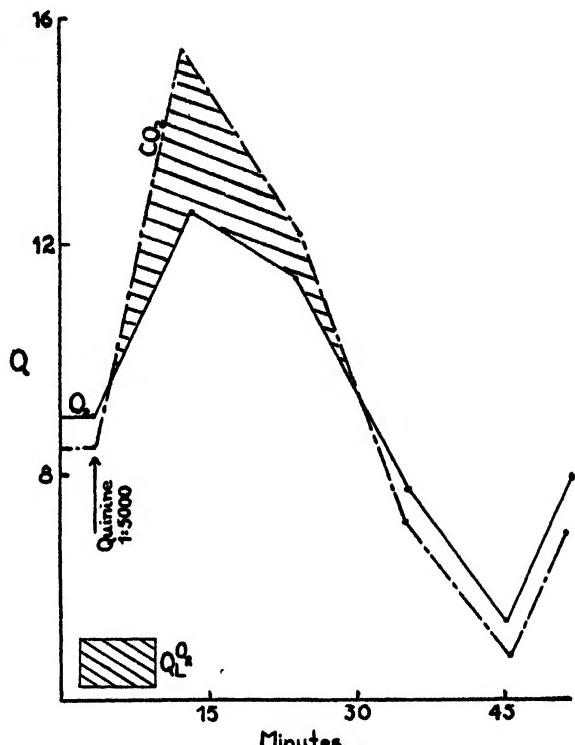
FIGURE 5
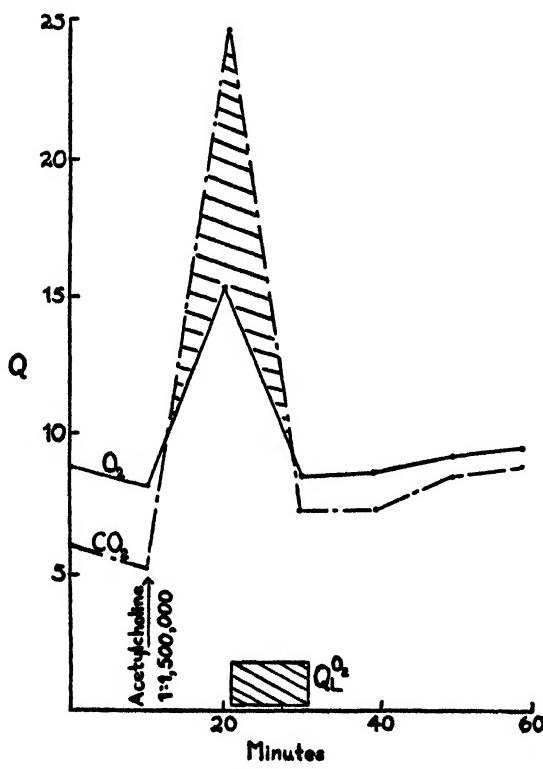
expectation, aerobic glycolysis may appear even though respiration is increased. This type of change in cell metabolism is possibly connected with changes in cell permeability. This would account for the fact that physiological stimulation of glands or fertilization or artificial activation of sea urchin eggs is accompanied by the same changes in cell metabolism as occur in the case of injury. In all these cases changes in cell permeability seem to be associated with the cause of the changes in metabolism.

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FIGURE 4

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DISCUSSION

Dr. Hoagland: In connection with the stimulating effect of cyanide on cell respiration, has any work been done indicating an increased permeability of cells associated with that early stimulation before the inhibition sets in?

Dr. Fleischmann: I know of no work which has been done in this connection.

Dr. Barker: I should like to bring up another theory of convulsions in contrast to the one which you mentioned, growing out of the work of Himwich with insulin. He finds that when the glucose concentration of the blood is greatly decreased, practically no substrate is available to the brain to be acted upon. Its metabolism therefore decreases to very low levels. Under these conditions convulsions are produced. Himwich has been able to duplicate the same end result by producing anoxemia from inhalation of nitrogen, so that convulsions in these instances seem to result from a decreased metabolism of brain rather than an increased metabolism.

Dr. Fleischmann: I quite agree that this effect is not specific for the increase of available nutrients. It is possible that convulsions can be brought about by any sudden change in the amount of nutrients available to the brain.

Dr. Warren: I would like to comment on the experiments of Fujita on bone marrow, which you quoted. He showed that Ringer solution damaged rat bone marrow cells so that aerobic glycolysis became very large and respiration very small. I am a strong proponent of the view that one should work in serum in tissue metabolism studies, but at the same time this effect of Fujita's is not universal with bone marrow. In agreement with Orr and Stickland, I have found that one can work in Ringer's solution with rabbit bone marrow without there being much of a falling off of respiration or increase in aerobic glycolysis. Such aerobic glycolysis as there is, moreover, persists in serum although in this medium both respiration and glycolysis are about 70 per cent higher. I doubt if one can make the generalization that aerobic glycolysis always means injury.

In the metabolism in leucemia we have a confusing state of affairs; in some types of leucemia there is no aerobic glycolysis, but in other types there definitely is. I refer particularly to the experiments of Victor, who has followed the changes in metabolism in mouse leucemia, and has shown that the aerobic glycolysis is considerable, particularly in certain malignant forms. The metabolism is also influenced by the host, and the malignant type of metabolism occurs before there are histologically demonstrable evidences of malignancy in the cell.

Dr. Fleischmann: I have shown these data from Fujita's paper because they show such a marked decrease of respiration. It is quite possible that this is because Fujita did not use such careful methods of isolating the bone marrow as Warren has used in his work. But I think that this experiment of Fujita can be interpreted as an example of respiration being more sensitive to injury than glycolysis. This is shown by plotting respiration and anaerobic glycolysis against time, and is quite independent of the question as to whether bone marrow shows aerobic glycolysis under physiological conditions.

As to the leucemic cells, I think that Kempner's last paper shows very clearly that if you have very young cells and treat them very carefully, you find that they have practically no aerobic glycolysis. Even if you do not agree to the clear-cut picture I drew in following Kempner's ideas and my own, I think you will agree that the measurements of metabolism of young blood cells do not suggest malignancy.

Mr. MacLeod: Recently, Rhoads and I confirmed Fleischmann's results on the exudate leucocytes of the rabbit, but I am not convinced that the very high aerobic glycolysis shown by these cells is due entirely to injury.

First of all, it should be made clear that the leucocytes obtained from the rabbit peritoneal exudate are predominantly of one type, namely "polymorphonuclears". Secondly, cells of this type in the rabbit are not true neutrophils but are pseudo-eosinophils, morphologically quite different from the neutrophil cells of the goose, the rat and

man. Fleischmann has given evidence that leucocytes obtained directly from blood do not show the high aerobic glycolysis and concludes from this that leucocytes *in vivo* do not have aerobic glycolysis and that it is in their passage from the blood stream into the peritoneal cavity that they acquire aerobic glycolysis, as a result of some injury.

Fleischmann's evidence is not conclusive, for several reasons. The results obtained from cells taken directly from the blood of different species show that leucocytes have a very small aerobic glycolysis, but there are no figures to show what percentage of the cells are neutrophils. In few species does the percentage of neutrophils exceed 55 p.c. and in many animals the percentage is considerably lower than 55 p.c. Until it is shown that neutrophils, lymphocytes, monocytes, etc. all have the same type of metabolism, we cannot conclude that results from the aggregate represent the metabolism of one type. Thus it is possible that all the aerobic glycolysis may be due to one class of cell (*e.g.*, the neutrophil) and if there are relatively few neutrophils present in a given specimen of blood then the aerobic glycolysis figure for the neutrophil may really be quite high.

Furthermore, no experiments have been done on cells obtained directly from the blood of the rabbit. Here again, it would be necessary to separate the different types of leucocytes and measure their metabolism separately before it could be shown that the rabbit neutrophils *in vivo* have no aerobic glycolysis. This would be difficult, since in my experience with rabbits the normal percentage of neutrophils in the blood seldom exceeds 35 p.c. and at times is as low as 18 p.c.

The term "injury" as used in Fleischmann's paper is rather hard to define, as he himself has shown that the exudate leucocytes *in vitro* remain capable of carrying out the functions ascribed to them *in vivo*. Their phagocytic properties are largely retained, and Fleischmann has shown that this function depends on glycolysis and not on respiration. Again, the term "injury" would seem to imply an irreversible process. In the case of exudate leucocytes, the Q_{O_2} in serum is from 35 p.c. to 100 p.c. higher than in Ringer-phosphate. Thus, the respiration can be improved markedly. The aerobic glycolysis in serum is difficult to measure accurately because of the CO_2 retention of serum, but these cells still show a high aerobic glycolysis in serum; even the respiration is markedly improved in the same medium.

I have emphasized the case of the rabbit exudate leucocyte first because in Fleischmann's paper the cell is offered as a good example of the "injured" cell. Secondly, the aerobic glycolysis is very much higher (about 4 times) than the aerobic glycolysis of any other "injured" tissue offered as an example by Fleischmann. Until it is demonstrated that this high aerobic glycolysis is due

to injury, I would prefer to think that the rabbit exudate leucocyte is a unique cell and worthy of further investigation.

Dr. Fleischmann: I quite agree that rabbit exudate cells are unique cells. But Kempner and Peschel have found very similar changes in human leucocytes in artificially produced blisters in the skin. I am quite convinced that the exudate cells in MacLeod's experiments are young cells, but it is not inconceivable that they undergo some change or damage in the process of migrating into the exudate. Experiments in which both respiration and aerobic glycolysis of cells from the bone marrow, from exudates, and from blood are compared simultaneously might yield further information.

Mr. MacLeod: The cells of the rabbit exudate are obviously derived from the circulation. Ponder and I have shown that an exudate taken about 15 hours after injection of saline contains more leucocytes than were present in the circulation at the time of injection of the saline. Some of the cells in the exudate are therefore cells very recently liberated by the bone marrow and can truly be called young cells. Further, we have shown that a rabbit subjected to repeated exudates furnishes, in the later exudates, a leucocyte whose respiration is much higher than that from the earlier exudates. Since the leucocytes in the circulation showed increasing immaturity and since only these cells can appear in the exudate, we concluded that the younger cells had a higher respiration. This conclusion is not in harmony with that of Kempner, who has shown that young myeloid cells from human myeloid leucemias have no higher a respiration than the mature neutrophils.

Unfortunately we did not measure the glycolysis of the young cells in the rabbit.

Dr. Ponder: It seems to me that there is one important point which has to be taken into account in all studies of this kind. We measure O_2 consumption, CO_2 output, etc., and express the results in mm.^{3}/10^6 cells/hr., say. But in comparing two samples, one taken under one set of circumstances and the other taken under another, or one taken before some kind of experimental interferences and the other taken after, we do not usually know that the volume of the cells in the second sample is the same as that in the first, and so the second sample, although equal to the first as regards number, may differ from it as regards cell volume, and differences in Q_{O_2} may be merely reflections of volume differences. As an example, the apparent increase in oxygen consumption which MacLeod and I obtained for the cells of the peritoneal exudates of rabbits after several exudates may have been a reflection of an increase in cell volume, for the cells were obviously larger. Unfortunately, volume measurements are not easy to make, and we did not make them. And, after

all, is it the volume of the cell which determines the oxygen consumption? It might be the surface, the nucleus, or anything else you care to think of. In most work of this sort we use as a unit mm.^{3}/mg./hr. Now, why "per milligram"? Why not "per unit surface", "per unit nuclear volume", or per anything else that you choose to suggest? There is no answer, as far as I can see, and yet the problem is fundamental.

Dr. Fleischmann: This is certainly a very important point in the whole problem of measuring cell metabolism. The definition of Q_{O_2} as oxygen consumption per mm.^{3}/mg./hr. was introduced by Warburg, and I could think of no other unit to be used for expressing the oxygen uptake of tissue slices. For isolated cells another term may be more adequate. But in comparing oxygen uptake of different cells and tissues, Q_{O_2} may still be the most acceptable unit.

Dr. Shorr: I am glad to hear you emphasize the necessity for caution in interpreting the change in respiration which occurs as a result of injury, in that it may be due to some specific injury, but it may also be associated with accumulation of substrate. In the removal of muscle, for example, you could get curves of the type you indicate, apparently associated with the temporary accumulation of lactic acid, and sometimes such experiments have been confusing in that one has found oneself unable to get stimulation until one has washed out the accumulation of lactate. This error, I think, has been fallen into from time to time in attempting to correlate *in vitro* respiration with the same type of respiration in the whole organism. I remember some experiments of Gerard, in which he attempts to show that it is possible to approximate the rate of respiration of the cortex *in vitro* if one chooses a short period of the beginning of the experiment and extrapolates back to the time of removal. If one estimates the lactic acid present initially, one finds that it may be the reason for the higher respiration initially, subsequent diffusion and neutralization bringing the respiration down.

Dr. Cori: I wonder whether you would subscribe to the idea that injury abolishes the effect of respiration on fermentation, that is, that injury inhibits the Pasteur reaction.

Dr. Fleischmann: It is quite probable that this is the mechanism of the effect of injury on cell metabolism. I remember that Warburg suggested this explanation of my results on leucocytes.

Dr. Korr: Did I understand you to say that you were in agreement with the hypothesis that the increase in respiration might be due to the increase in permeability?

Dr. Fleischmann: Yes; to be more exact, in changes in permeability.

Dr. Korr: Although increases in permeability may be almost invariably associated with increases

in respiration, I think it is hazardous to draw conclusions as to the causal relationship between the two. In the case of the sea urchin egg, the increase in respiration on fertilization is certainly not attributable to increased permeability to diffusible nutrient substances, since all the nutrients are already inside the cell. The experiments of Shapiro on egg halves separated by centrifuging are also evidence against this hypothesis. The respiration of the light half only is increased on fertilization, despite the fact that both halves undergo, presumably, the same surface changes. I think there is no clear-cut case in which the rate of penetration of substrate controls the rate of respiration.

Perhaps a more likely hypothesis is that the cell structure or the cell membrane is so related, or connected, to the intracellular structure that a change in the surface organization, whether it be brought about by injury, fertilization, surface active agents or something else, will bring about changes in intracellular organization, reorientations of intracellular surfaces, changes in relations between enzymes, between enzymes and substrates, etc. That such changes take place in sea urchin eggs is already well known. They are being repeatedly demonstrated on other cells and tissues, including skeletal muscle, certain glands, smooth muscle, etc. Changes in respiratory rate, glycolysis, respiratory quotient and the catalytic systems involved, are now known to take place as a consequence of certain changes at the cell surfaces. The permeability to diffusible substances in these cases is certainly not the controlling factor. The changes in permeability are merely another corollary of changes in the organization of the cell surface, and are not the cause of the metabolic changes.

Dr. Fleischmann: It may be that we ought to discard the term "change in permeability" as being too general.

Dr. Korr: I wonder, in view of the fact that the Pasteur reaction occurs only in intact cells, why these factors have not been taken into consideration, and why the importance of cell structure and organization have been so completely neglected with respect to this phenomenon. Is it not quite possible that low O_2 tension or anaerobiosis may bring about reversible changes in cellular organization, in the cytoskeleton, if you like? These, in causing changes in accessibility of enzymes to substrates and changes in the spatial relations of enzymes, would also cause marked functional changes. Certainly such phenomena are not without precedent. Could they not be an important part of the mechanism of the Pasteur reaction?

Dr. Velick: The relative roles of permeability and internal cellular structure upon metabolism may become accessible to investigators of such

organisms as the slime mold, which is ameboid, relatively simple in structure, yet large enough to be handled as a tissue. Seifriz has studied the effects of various chemical and physiological agents upon such properties as protoplasmic streaming and viscosity, using micromanipulation techniques. Many of the changes are microscopically visible and can be followed progressively with the microcinematograph. Marked changes have been observed after treatment with quinine, and this is of particular interest because you have said that quinine in some cases causes an increase in oxygen consumption. What tissues have been treated with quinine in respiration studies?

Dr. Fleischmann: The experiments I mentioned were done on liver tissue and on salivary glands.

Dr. Velick: But not on blood cells?

Dr. Fleischmann: Not to my knowledge. The influence of quinine on blood cells has certainly not been studied by the same methods as were used for the tissues just mentioned.

Dr. Velick: The information on blood would be of particular interest because after an animal infected with malaria is treated successfully with quinine the parasite in the red cell is observed to degenerate. Every stage of the parasite is not equally affected, but only certain stages in its life cycle, and these vary with the species of malaria and the anti-malarial agent used. One is impelled to ask whether the specificity of the drug for various stages of the parasite is due to progressive changes in permeability of the host red cell caused by the growing parasite, or whether the drug alters the metabolic activity of the red cell host or the parasite during one of its stages. During the life cycle of many microorganisms large changes in the type of metabolic activity occur. An indication that such factors may be involved in malaria is further offered by the fact that in some forms of the disease the young parasite invades the actively respiring reticulocyte rather than the mature red cell. We have begun respiration studies of parasitized and normal cells to see if we can throw light on some of these factors.

Dr. Shapiro: Concerning some of Korr's remarks, I shall have more to say later in this Symposium. But I would like to point out that both his and Runnstrom's experiments have shown clearly that it is not the change in permeability in the cell which controls the alteration of respiration on fertilization; it is definitely an alteration in the availability of the chemical material inside of the cell. Did I understand you to say that one of the features of fertilization is necessarily an increase in oxygen uptake? That was an idea proposed by Loeb for the sea urchin egg.

Dr. Fleischmann: I think it is in the special case of the artificial activation of the sea urchin egg; it may be so in a number of other cases. But I agree with you that it is not a general principle.

Dr. Shapiro: There is at least one exception, and that is in the case of fertilization of the egg of the worm *Chaetopterus*, where there is no increase in respiration on fertilization, but actually a decrease.

Dr. Fleischmann: Is there a change in permeability also?

Dr. Shapiro: There is no appreciable change in water permeability as determined from preliminary experiments, but there is a drop in respiration.

Dr. Stannard: One case parallels those which have been mentioned and is worth bringing up. In some work on yeast done by Stier and myself, the intact yeast, in the absence of added substrate, seems to use its internal stores by a purely oxidative process. There is no alcohol production and no CO_2 production under anaerobic conditions, and I think Smythe has obtained results by chemical analysis which support this general observation. But as soon as the yeast is ground with sand, pressed, or otherwise injured, immediately the glycogen sources are fermented and anaerobic CO_2 production starts. If glucose is present in the medium fermentation proceeds aerobically or anaerobically, so this phenomenon is limited to the utilization of the intracellular stores.

There is one question about the experiments with liver cells which is not quite clear to me. Are those values for the Q_{CO_2} aerobic or anaerobic?

Dr. Fleischmann: They are aerobic.

Dr. Stannard: Are measurements available for the anaerobic CO_2 production?

Dr. Fleischmann: No. In these series of experiments anaerobic glycolysis was not measured.

Dr. Stannard: It seems to me that without measurements of the anaerobic CO_2 production it is hard to distinguish between a change in the type of metabolism and simply a stimulation of the total metabolism. For instance, in the case of muscle one could give a case which would be superficially analogous. In contracture caused by, let us say, potassium chloride, there is an increase in lactic acid production, and if one uses the same muscle for measurement as was used before contracture, one would notice what appears to be the beginning of anaerobic glycolysis; this is simply because now the demand for oxygen is so large that the muscle which was previously thick enough to allow complete diffusion of the oxygen is now too thick, and the center section is producing lactic acid anaerobically, whereas the surface sections are oxidizing the lactate as rapidly as they can. In that case there is an increase in all three factors, anaerobic glycolysis, aerobic glycolysis, and respiration; but that effect is completely reversible, and I do not think it is usually considered as an injury.

FERRIHEMOGLOBIN

Kurt Salomon

After half a century of scientific controversy it is today an accepted fact that methemoglobin is the true oxidation product of hemoglobin. The iron in its prosthetic group is in the ferric state, whereas the prosthetic group of hemoglobin contains ferrous iron. The oxidation requires one hydrogen equivalent for one gram atom of iron. This oxidation takes place in air even at low temperatures. Küster and Kimmich (1) report that crystallized oxyhemoglobin is slowly transformed into methemoglobin even when allowed to stand in the cold. It has been reported by Brooks (2) that this oxidation takes place even at —20° C. at a noticeable rate.

Hemoglobins are the only iron compounds known to be able to bind oxygen loosely in a reversibly dissociable manner. This faculty, moreover, belongs not only to the naturally occurring hemoglobins but also to those produced by the combination of chemically prepared hemins with native globin followed by reduction of the hemin iron (3). The presence of globin in these compounds is essential for the formation of oxygenated products in which the iron remains in the ferrous state rather than being oxidized to the ferric form. Hemoglobin-methemoglobin forms a reversible oxidation-reduction system of a normal potential of $+152$ millivolts at pH 7 (4). It is not surprising, therefore, that methemoglobin should be able to act as an oxidation catalyst under suitable conditions, provided that the reoxidation of its reduced form to methemoglobin takes place and no oxyhemoglobin is formed. Methemoglobin catalyses are interesting because their study may throw some light on the mechanism of the catalytic function of hemochromogens present in living cells.

The Problem of Step-wise Oxidation of Hemoglobin

The existence of intermediates

Since it has been demonstrated by Svedberg (6, 6a) and Adair (5) that the hemoglobin molecule contains four hemin groups, the problem arose whether the four iron atoms present have the same physical and chemical behaviour or whether in the oxidation of hemoglobin some of the iron atoms become oxidized before the others. In this case different types of oxidation, and consequently also of oxygenation products, should be detectable. The oxidation of oxyhemoglobin to methemoglobin not only consists in a valency change of the iron atom but also in a release of oxygen. One molecule of oxygen is set free per atom iron oxidized. Therefore the oxidation of four iron atoms should yield a compound of the type Hb_4, the oxidation

of three iron atoms a compound of the type Hb_4O_2, in which only one iron atom would be oxygenated, and so on. The possibility of the existence of such intermediate compounds has been investigated by Conant and his co-workers (7), Brooks (2) and others (8). Osmotic pressure measurements carried out by Adair (5) have been stated to support the view of a step-wise oxidation of hemoglobin and of a step-wise reduction of ferrihemoglobin. Conant and co-workers attempted to measure the solubility of the suggested oxidation products of hemoglobin, assuming that the various intermediates may have different solubilities, independent of each other. They were, however, unable to find any indication for the existence of stable intermediate oxidation products of hemoglobin. Moreover, as Conant later pointed out (9), the assumption of independent solubilities for the different molecular species seems not to be justified for substances of high molecular weight.

In a kinetic study of the oxidation of hemoglobin by molecular oxygen it has been found that the velocity of methemoglobin formation is dependent on the oxygen pressure up to about 200 mm. It is interesting to note that the highest velocity of oxidation takes place at a pressure of only 20 mm. (Fig. 1). As has been shown by Neill and Hastings (8) and by Brooks (2) hemoglobin, but not oxyhemoglobin, is oxidized. For the relation between oxygen pressure and the rate of reaction,
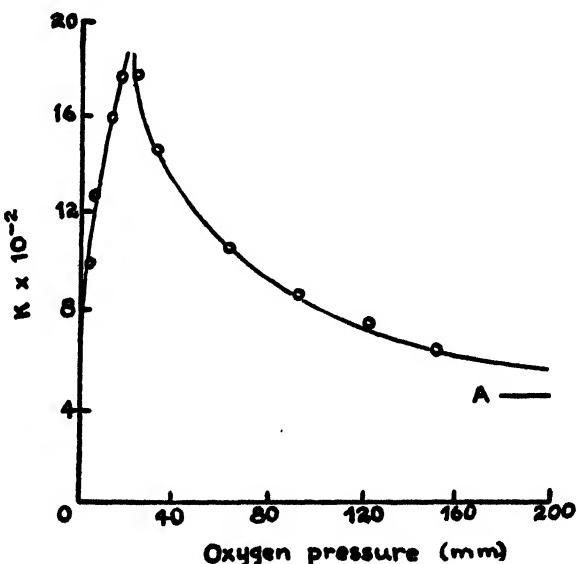
Fig. 1. Velocity constants at different oxygen pressures. A — value of k at 723 mm. O_2. Reprinted from Brooks (2).

Conant and Fieser (4a) suggest the following possible paths of reaction:

(1) the spontaneous decomposition of oxyhemoglobin into methemoglobin, O_2 and H_2O (or H_2O_2);

(2) the oxidation of reduced hemoglobin by oxyhemoglobin;

(3) the oxidation of reduced hemoglobin by oxygen.

In (1), the rate would increase with increasing oxygen pressure. Case (2) would hold only for a bimolecular reaction. However, the pseudo-monomolecular course of the reaction and the maximum rate of oxidation suggest qualitatively a reaction between oxygen and reduced hemoglobin (Case 3). In the equation $Hb + O_2 \rightleftharpoons HbO_2$ the equilibrium of the reaction is strongly shifted to the right. As more oxyhemoglobin is formed, less hemoglobin will be free to react. An increase of oxygen pressure increases the amount of oxyhemoglobin and shifts the equilibrium of the oxygenation reaction even further in favor of oxyhemoglobin. It has been shown that the rate of oxidation of hemoglobin by dissolved oxygen is maximal at low oxygen pressure. This is to be expected if only hemoglobin, and not oxyhemoglobin, is oxidized by oxygen to methemoglobin. Under the conditions of Brooks' experiments, besides the methemoglobin only hemoglobin or oxyhemoglobin of the type Hb_4O_8 could be found. It has been shown by Conant and later confirmed by Havemann and Wolff (10) and by Taylor (11) that four molecules of ferricyanide are required for the oxidation of one molecule of hemoglobin; in other words, that one electron is given off per iron atom. It is interesting to note that in the titration curve published by Havemann and Wolff, who worked with the method of oxidative and reductive titration, the slope and index potential correspond to $n = 1$. The value for the normal potential (E'_o) at pH 7 for the hemoglobin-methemoglobin system of $+90$ mv. is more negative than found by Conant and Pappenheimer ($+ 152$ mv.).

The slope of the E'_o/pH curve given by these authors can be satisfactorily explained by their hypothesis that at any pH above 6.2 the equation

$$Hb - 4\epsilon \rightleftharpoons \text{methemoglobin} + 4H^{\cdot}$$

holds, i.e., in this range one hydrogen ion is formed per electron given off by hemoglobin during the oxidation. A further indication of the identical chemical nature of the four Fe groups in the hemoglobin molecule has been brought forward by Warburg, Negelein and Christian (12). In a photochemical study of the problem whether the four iron-carbon monoxide groups in a carbon monoxide hemoglobin molecule dissociate to a different degree at the same light absorption, their measurements furnish no indication of a different photochemical behavior of the four Fe-porphyrin groups.

The problem of the existence of intermediate oxidation products of hemoglobin has been discussed lately by Haurowitz (13) from a different point of view. Crystallizing hemoglobin he obtained two different types of crystals: needles for oxyhemoglobin and rhombic plates for hemoglobin. He never was able to find any other form of crystal. If, therefore, intermediate oxidation products between hemoglobin and oxyhemoglobin can exist, they probably do not exist in a stable and crystallizable form. On the other hand it must be kept in mind that hemoglobin is by no means a uniform substance. It has been shown by Haurowitz (14) that horse hemoglobin contains at least two different species of molecules: one containing one atom of sulfur per atom of iron and the other two or more atoms of sulfur per iron atom. Evidently the causes for the existence of different hemoglobin molecules in the same species of animal have to be searched for in the globin component and not in the hemin group.

Ferri-ferro complexes

An interesting contribution to the possible existence of intermediates, containing simultaneously ferri- and ferro-radicals in a reversible oxidation-reduction system, has been made by Michaelis and Smythe (15). They observed that during the reductive titration of pentacyano-aquo complexes of iron, the violet color of the ferri-compound changes to gray, green and finally to weak yellow, which is the color of the completely reduced ferro-compound at the end of the titration. The color changes during reduction indicate that the iron complex compound passes through more than two oxidation-reduction levels. Between the totally oxidized ferric and the totally reduced ferrous form of the compound there exist three intermediate steps, in which some of the four iron atoms are in the ferric and some in the ferrous form.

Warburg has suggested that the respiratory ferment, like hemoglobin, may exist in reduced, oxidized, and oxygenated forms. These three compounds would be analogous to hemoglobin, methemoglobin and oxyhemoglobin. Whereas, however, most methemoglobins after reduction are not reformed by oxygen but need for their reoxidation a second reversible redox system, the reduced form of which is autoxidizable and the oxidized form of which reacts at an appreciable rate with ferro hemoglobin, the reduced form of the respiratory ferment is autoxidizable. Spectroscopical experiments of Warburg and his co-workers (16) indicate that this reoxidation leads through an unstable intermediate containing ferrous iron and

loosely bound oxygen. The formation of such a primary adduct is also suggested by the fact that carbon monoxide is capable of competing with oxygen for the ferrous iron of the respiratory ferment in a perfectly reversible manner depending on the partial pressure of the two gases.

The Catalytic Action of Methemoglobin
Hemin- and methemoglobin-catalyses

The catalytic power of hemins and hemochromogens has been studied in various systems. Kuhn and Meyer (17) investigated the oxidation of fatty acids in the presence of pyridine and hemin. Krebs (18) coupled various nitrogen bases with hemin and studied their catalytic activity in the oxidation of cysteine. The catalytic power of his substances depended greatly on the chemical constitution of the nitrogenous base. He found nicotine-ferri-hemochromogen to be the most active of the substances tested. It is worth mentioning that hemoglobin showed no effect in Krebs' experiments. Barron compared the catalytic value of a series of synthetic hemochromogens in the oxidation of linseed oil. The catalytic power of pilocarpine- and histidine-hemochromogens were found to be the highest of all the hemochromogens studied (19). In all the experiments mentioned ferrihemochromogen is reduced to ferrohemochromogen, the latter being reoxidized by oxygen.

Methemoglobin in non-nucleated red blood corpuscles

The catalytic oxidation of glucose by various hemins in the presence of mammalian erythrocytes has been investigated by Warburg and Kubowitz (20). The most active preparation was phaeo-phorbid-a-hemin, a green hemin closely related to chlorophyll. The mechanism of this catalysis consists in the catalytic formation of methemoglobin, which in turn burns the substrate. This reaction can be written in the following manner, according to Warburg's concept of the hydrogen transport in the cell:

$$O_2 \underset{\text{MB*}}{\overset{\text{Hemin}}{\longrightarrow}} Hb \rightleftharpoons Methb \longrightarrow$$

$$\underbrace{\text{Coferment II} \rightarrow \text{Robison ester}}_{\text{``Zwischenferment''}}$$

or in more detail:

$$O_2 \underset{\text{MBH}_2 \rightleftharpoons \text{MB}}{\overset{Fe^{\cdot\cdot} \rightleftharpoons Fe^{\cdot\cdot\cdot}}{\longrightarrow}} Fe^{\cdot\cdot} \rightleftharpoons Fe^{\cdot\cdot\cdot} \rightarrow$$

$$\underbrace{Py(PO_4)_3H_2 \longrightarrow \text{Hexosephosphate}}_{\text{Protein of the hexosephosphate-dehydrogenase}}$$
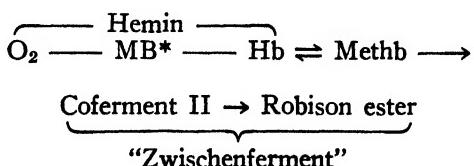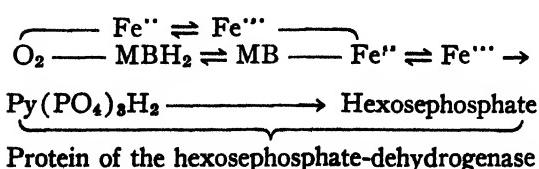
* Methylene blue as an example of a reversible dyestuff.

In this catalysis an autoxidizable redox system containing iron, namely, heme-hemin, oxidizes another iron compound, hemoglobin, which is not autoxidizable. In comparison of this catalysis with oxidations taking place in the living cell, the hemin plays the role of the respiratory ferment whereas the hemoglobin-methemoglobin system may be compared with the cytochrome system. One would expect that the catalytic efficiency of the hemins is proportional to the amount of methemoglobin formed. This is not the case. Pyrrohemin, for instance, produces less methemoglobin than blood hemin does, but has a higher catalytic effect. Warburg's explanation for this phenomenon is the assumption that the different methemoglobins are produced at different places in the cell, and that only the methemoglobin on the surface of the cell acts as a catalyst. It seems, however, more probable that methemoglobins produced by different chemical agents exhibit differences in their chemical structure which would explain unequal catalytic efficiency.

Hemins, in a similar fashion as methylene blue and other oxidation-reduction systems, stimulate the respiration in mammalian erythrocytes only in intact cells. In hemolyzed cells none of the substances mentioned enhances the respiration because the phosphorylating system has been destroyed. The addition of hexosemonophosphoric acid, however, allows the stimulation of respiration to take place even though the structure of the cells remains destroyed (21).

Methemoglobin catalyses take place only in the presence of a substrate, for instance glucose, which is oxidized by methemoglobin and in the presence of a second autoxidizable oxidation-reduction system which reoxidizes hemoglobin to methemoglobin. Whether this second catalyst is truly autoxidisable, like the respiratory ferment, or whether its reoxidation only takes place in the presence of heavy metals, like the reoxidation of leucomethylene blue to methylene blue in a certain pH range, is irrelevant as long as its reoxidation takes place at least at the same velocity as the reduction of methemoglobin. Obviously those methemoglobin catalyses are more significant in which the reoxidation of hemoglobin is performed by another iron porphyrin compound because they are similar to the mechanism of cellular respiration; namely, the oxidations proceeding through the cytochrome-cytochrome oxidase system.

It was shown first by Meyerhof (22) that methylene blue induces respiration in cells whose respiratory system has been damaged. Barron and Harrop (23) found later that methylene blue has the same effect in weakly respiring cells like mammalian erythrocytes or echinoderm eggs. Mammalian erythrocytes produce their energy practically by glycolysis alone, their respiration

being small. After the addition of methylene blue to the cells, the respiration is greatly increased. The mechanism of the reaction may be represented according to Warburg by the following equations:

$$MB + 2\,Hb \rightleftharpoons MBH_2 + 2\,Methb$$

$$2\,Methb + CoH_2 \rightleftharpoons 2\,Hb + Co*$$

$$MBH_2 + O_2 \rightleftharpoons MB + H_2O_2$$
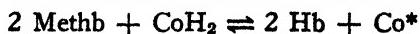
Methylene blue oxidizes hemoglobin to methemoglobin, which in turn oxidizes carbohydrate through the intermediation of the pyridine-nucleotides. The leucomethylene blue is reoxidized to methylene blue by molecular oxygen. Without methylene blue present one molecule of methemoglobin can act only stoichiometrically as an oxidant for the substrate; this oxidation must necessarily come to an end as soon as all the methemoglobin is reduced.

It has been shown by Barron and Hoffman (24) for sea urchin eggs, and by Michaelis and Salomon (25) for rabbit erythrocytes, that other dyes besides methylene blue are able to stimulate cellular respiration. The increase of the respiration of sea urchin eggs was found to be related to the normal potential of the dye, the highest effect being at 0 volt at pH 7. More positive as well as more negative dyes were less efficient. In the experiments with mammalian erythrocytes, on the other hand, a general parallelism could be shown between the stimulation of respiration, the normal potential of the dye and its faculty to produce methemoglobin. In the case of certain dyes with a negative normal potential no methemoglobin formation could be detected although some stimulation of respiration was observed. We expressed the ability of the dye to form methemoglobin by the percentage of the amount of oxygen freed in a parallel experiment carried out with potassium ferricyanide in an identical blood sample. The amount of methemoglobin formed was measured by the increase of gas pressure in the Warburg microrespirometer, due to the liberation of released oxygen. The pressure increase represents all of the oxygen capable of being liberated only when the reduced form of the oxidizing agent is not autoxidizable (*e.g.* ferrocyanide). In this case one mol of oxygen appears per mol methemoglobin formed. If, however, as an oxidant a reversible dye is used with a potential high enough to oxidize all the hemoglobin present, it will consume for its reoxidation a part of the oxygen developed. Phenol-indophenol, for instance, has a redox potential of $+250$ mv. at pH 7. In the case of this dye one expects the following quantitative relationship:

$$1/2\,mol\;PI* + 1\,mol\;HbO_2 \rightarrow$$
$$1\,mol\;methb. + 1/2\,mol\;LPI** + 1\,mol\;O_2$$

$$1/2\,mol\;LPI + 1/4\,mol\;O_2 \rightarrow 1/2\,mol\;PI$$

$$\overline{1\,mol\;HbO_2 \rightarrow 1\,mol\;methb + 3/4\,Mol\;O_2}$$
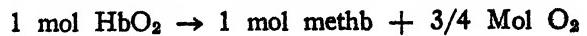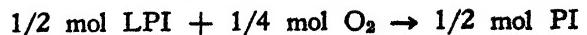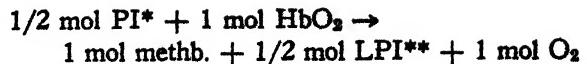
Half a mol of phenol-indophenol oxidizes one mol of ferrohemoglobin to ferrihemoglobin. Half a mol of leucophenol-indophenol consumes a quarter of a mol of oxygen for its reoxidation. Therefore only three quarters of the oxygen developed by potassium ferricyanide can be expected when a reversible dye of a suitable potential is used for the oxidation of hemoglobin. If the amount of oxygen developed by potassium ferricyanide is 100, we expect 75 using phenol-indophenol. Our experimental value was 80, which is within the limits of the method. The molar concentration of the dyes used in our experiments was approximately 100 times that of the hemoglobin present.

The case of methylene blue demands special consideration. In the reaction of equivalent concentrations of hemoglobin and methylene blue under anaerobic conditions, in accordance with the potential range of the two systems, the reaction comes to equilibrium when about 10 p.c. of the hemoglobin is oxidized (26). This, however, does not hold in the presence of oxygen. In this case the leucomethylene blue formed by reaction with hemoglobin will be reoxidized to methylene blue by O_2 and re-enter the oxidation cycle. Therefore the amount of oxygen released by the use of methylene blue as an oxidant of hemoglobin is considerably higher than the value observed in the absence of oxygen. Michaelis and Salomon have studied the stimulation of the respiration of mammalian erythrocytes suspended in 0.9 p.c. sodium chloride by the dyes used for the formation of methemoglobin in solutions of oxyhemoglobin. Dyes of a very negative potential which are not reversibly reducible, or reducible only with great difficulty, have no influence on the respiration of red blood cells. A maximum effect was found in the potential range of methylene blue and could not be increased by the use of dyes of a higher normal potential. Barron and collaborators were able to show that in sea urchin eggs, which do not contain hemochromogens, as well as in mammalian erythrocytes in which the iron containing system was inhibited, dyes are able to oxidize directly the substrate, a reaction not involving the formation of methemoglobin. From these experiments, it can be concluded that two different mechanisms are able to stimulate cellular respiration: the oxidation of the substrate by methemoglobin produced by the oxidative power of a sec-

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* Co = Coenzyme II

* PI: Phenol-indophenol.
** LPI: Leucophenol-indophenol.

ond redox system and/or the oxidation of the substrate directly by the dye. It was shown (26) that both mechanisms take place at the same time in non-nucleated red blood corpuscles when methylene blue is used as a stimulatory substance for their respiration.

The catalyses produced by methylene blue or other dyes in red blood corpuscles are only models for biological oxidations in so far as they are oxidations by porphyrin bound iron, in which reversible dyes replace the respiratory ferment. Nearer to physiological conditions is the oxidation of hemoglobin by hemin.

Besides the formation of methemoglobin by green and red hemins and redox dyes, the mechanism of its formation by phenylhydroxylamine, phenylhydrazine and amyl nitrite has been investigated in detail in Warburg's laboratory (27). The peculiar chemical reactions of some of the methemoglobin formed led Warburg to suggest the existence of differences in their chemical structure.

Phenylhydrazine acts as a methemoglobin forming agent by producing hemin which in turn oxidizes ferrohemoglobin. Phenylhydrazine splits the hemoglobin molecule into hemin and denatured globin. The hemin thus formed oxidizes the hemoglobin iron to the ferric state. The methemoglobin finally oxidizes the substrate with the aid of the *"zwischenferment"* system already mentioned. We deal here again with a catalysis in which an autoxidizable iron compound, heme, oxidizes a non-autoxidizable one, hemoglobin. These experiments are similar to those in which the addition of hemins to mammalian erythrocytes stimulates their oxygen uptake. That the mechanism of action of phenylhydrazine goes through the formation of methemoglobin has been shown in experiments in which so called "phenylhydrazine-cells" are shaken in the absence of glucose; the methemoglobin accumulates and can be seen spectroscopically. The formation of methemoglobin by phenylhydroxylamine supplies important evidence for the existence of different ferrihemoglobins. The difference in its chemical behavior as compared with methemoglobin formed by other reagents consists in its reaction with oxygen. Mammalian erythrocytes treated with phenylhydrazine are brown because of the formation of heme and its oxidation product hemin. That is the reason why these cells stay brown even after they are shaken with air.

Mammalian blood corpuscles treated with phenylhydroxylamine are also brown; this brown color, however, is due to the methemoglobin present. In this case no white precipitate appears due to the splitting of the molecule as in the case of phenylhydrazine. Phenylhydroxylamine probably forms hydrogen peroxide, which in turn oxidizes hemoglobin. When these brown cells are shaken with air in the presence of glucose, they do not turn red as a result of the formation of oxyhemoglobin as expected, but stay brown because the hemoglobin is re-oxidized to methemoglobin by oxygen instead of being oxygenated. Therefore not only another methemoglobin seems to be formed but also another hemoglobin. Warburg, on the basis of the following observations, suggests that this hemoglobin *in statu nascendi* is not oxygenated but oxidized. When blood corpuscles treated with phenylhydroxylamine are shaken in an oxygen free atmosphere in the presence of glucose the methemoglobin formed is reduced to hemoglobin. Subsequent treatment with air now produces the formation of oxyhemoglobin and the catalysis finds its end.

The methemoglobin formed by phenylhydroxylamine is the only methemoglobin acting as a true catalyst, its reduced form, hemoglobin, being re-oxidized by molecular oxygen, as opposed to the methemoglobins formed by hemins or reversible dyes, which act as auxiliary catalysts.

Amyl nitrite produces methemoglobin which is not catalytically active but which oxidizes glucose stoichiometrically; the hemoglobin formed is easily oxygenated. Warburg claims, however, that amyl nitrite-methemoglobin is different from other methemoglobins, assuming that it contains oxygen, not loosely bound as in oxyhemoglobin but chemically more reactive. The chemical constitution of this compound is unknown. It may possibly represent a complex compound of a peroxide with methemoglobin.

It seems necessary to look for the differences in the chemical behavior of various methemoglobins in the reactions between the methemoglobin forming agent and the hemoglobin molecule. It is known that methylene blue and other dyes react with globin; this reaction is undoubtedly oxidative in character, but the chemical nature of the groups reacting in the protein remains to be established. The reaction of ferricyanide with the protein part of the hemoglobin molecule has been investigated by Schueler (28). He measured manometrically the amount of carbon monoxide liberated from carbon monoxide hemoglobin upon the addition of potassium ferricyanide. When the oxidation of the ferro- to the ferri-iron in hemoglobin is measurable manometrically by the amount of oxygen developed, a further oxidation of the molecule taking place at the same time would not be noticed by the use of this technique, for no gas would be developed or absorbed. Schueler determined, therefore, in a parallel experiment, the quantity of ferricyanide reduced by means of iodometric titrations and found that more ferricyanide disappeared than was necessary for the amount of carbon monoxide developed. Separating the globin part from the prosthetic group he found that

each globin molecule reduces two molecules of fer-
ricyanide. The ferricyanide probably oxidizes the
SH-groups of the globin molecule, which after the
reaction fails to give a nitroprusside test. The
degree of oxidation of other groups in the globin
molecule may depend on the oxidizing agent used
and on the time during which the globin part and
the oxidant are in contact with each other. Re-
cently Mirsky and Anson (29) have shown that
oxidizable groups other than SH-groups are pres-
ent in various proteins which are capable of react-
ing with potassium ferricyanide, but the chemical
nature of these groups is not known.

Spectroscopically no differences have yet been
demonstrated between methemoglobins produced
by different agents. Methemoglobin is spectro-
scopically identified by the shift of the band in the
red from 630 mμ to about 610 mμ after adding
sodium fluoride at ca pH 6 (30). Although no
spectroscopical differences could be found under
these conditions it is possible that this test is not
sensitive enough to detect slight differences. A
more senstive criterion might be the catalytic prop-
erties of the methemoglobins formed by different
agents.

Formation of Methemoglobin in Biological Systems

It has been shown by Bernheim and Michel
(31) that mammalian tissues are capable to a
varying degree of forming methemoglobin. Phos-
phate extracts of the tissues were mixed with a
solution of hemoglobin and the amount of methe-
moglobin formed was determined. Kidney showed
the highest potency to form methemoglobin, fol-
lowed by heart, liver, brain, and muscle. Under
the experimental conditions the methemoglobin
formed showed no catalytic activity.

That organ extracts added to mammalian eryth-
rocytes stimulate their respiration has been shown
by Michaelis and Salomon (32) and has been con-
firmed by various authors (33, 34, 35, 36). The
substance present in organ extracts which in many
respects has an effect similar to that of methylene
blue was called the "respiratory supplement".
Like methylene blue the respiratory supplement
stimulates the respiration of non-nucleated red
blood corpuscles but is without influence on the
oxygen uptake of fowl erythrocytes. The organs
containing the largest amount of this substance are
liver and kidney. Muscle, brain, and blood serum
contain very little. We have not been able to iso-
late the substance and until now its chemical na-
ture is little understood. It can not be excluded
that the respiratory supplement produces methe-
moglobin in the red cell and that this catalysis,
therefore, is also a hemin catalysis. It is impos-
sible to prepare organ extracts in which not even
traces of methemoglobin are present. Spectro-

scopically, however, no indication of the formation
of methemoglobin in the cells or in the organ ex-
tracts could be found. Very small amounts of
methemoglobin formed might have escaped detec-
tion with this method, because of its relative in-
sensitivity. The attempts made to identify the
chemical nature of the respiratory supplement in-
dicate that the substance is not identical with
methemoglobin. Adsorption and elution tech-
niques failed to concentrate the supplement.
Whereas freezing does not impair the activity of
the supplement, heating above 50° C. destroys it
and extensive shaking damages it greatly.

The effect of the various agents on mammalian
erythrocytes discussed above is summarized in
Table I.

Significance of Methemoglobin Formation for the Study of Organs in Vitro

The appearance of methemoglobin in the per-
fusion liquid of isolated organs, accompanied by
its denaturation and precipitation, restricts the
perfusion time for in vitro experiments to a mat-
ter of hours.

The best method available at present for the
study of whole organs in vitro is the Carrel-Lind-
bergh perfusion technique. Under sterile condi-
tions it is now possible to keep organs functioning
several weeks in the Lindbergh perfusion pump at
37° C., provided that the oxygen requirements of
the perfused organ are satisfied. This condition
can be fulfilled, however, for only a few organs,
such as the thyroid gland of the cat, dog, or rab-
bit. Enough oxygen is dissolved physically in the
circulating salt solution to supply the needs of
these organs, but the amount of oxygen thus car-
ried is insufficient for other organs. For the cul-
ture in vitro of liver or kidney, for instance, the
presence of oxygen carrying pigments in the per-
fusion liquid is imperative.

In 1938 Bing (37) perfused dog kidneys with
perfusion liquids containing the respiratory pig-
ment of the horseshoe crab (Limulus polyphe-
mus), hemocyanin, as an oxygen carrying sub-
stance. Hemocyanin as an oxygen carrier has
several advantages over hemoglobin for the perfu-
sion of organs. Metabolites produced by the or-
gan with a potential high enough to produce
methemoglobin are unable to produce methemo-
cyanin, the cupric form of hemocyanin. The oxi-
dation of hemocyanin, the normal potential of
which is +540 mv. at pH 7, can be performed
only by reagents of an extremely high oxidative
power like cerisulfate or potassium-molybdicya-
nide. Furthermore an oxidation of hemocyanin to
methemocyanin does not impair its function as an
oxygen carrier, because both the reduced and oxi-
dized compounds, hemocyanin and methemocy-
anin, are able to combine with oxygen reversibly.

TABLE I

Effect of Various Agents on Mammalian Erythrocytes *in Vitro*

(R.S.: Reducing system = coferment II, protein, hexosemonophosphate. D: dye.)

Reagent	E'_0 mv. pH 7	Methemoglobin formed* (p.c. of total Hb present)		Respiration increase*	Mechanism of reaction	Notes	References
		Intact cells	Hb. sol.				
Amyl nitrite	—	50	—	12×	Methemoglobin→R.S.	Agent washed out before respiration is measured.	(27)
Phenylhydroxylamine	—	45	—	18×	O₂→hemin→methemoglobin→R. S.	Agent washed out before respiration is measured. True catalysis.	(27)
Phenylhydrazine	—	36	—	18×	O₂→hemin→methemoglobin→R.S.	Agent washed out before respiration is measured.	(27)
Pyrrohemin	—	0	7	10×	O₂→pyrrohemin→methemoglobin→R.S.	—	(20)
Phaeophorbid-a-hemin	—	17	—	20×	O₂→phaeophorbid-a-hemin→ methemoglobin→R.S.	—	(20)
Phenol-indophenol	+250	—	80 p.c.	ca.15×	O₂→D→methemoglobin→R.S.	—	(25)
Chlorphenol-indophenol	+233	—	90 p.c.	ca.15×	O₂→D→methemoglobin→R.S.	—	(25)
Gallocyanin	+21	—	40 p.c.	ca.15×	O₂→D→methemoglobin→R.S.	—	(25)
Methylene blue	+11	—	—	15×	O₂→D→(methemoglobin)→R.S.	Suspension of rabbit erythrocytes used.	(27)
Methylene blue	+11	—	—	30×	O₂→D→(methemoglobin)→R.S.	Suspension of rabbit erythrocytes used.	(27)
Methylene blue	+11	—	53 p.c.	—	—	—	(27)
Respiratory supplement	—	—	—	ca.15	Not known	Extracts of liver and kidney show strongest effects.	(32)

* The concentrations of the agents used in these experiments may be found in the original papers.

Finally it has been shown by Millikan (38), that hemoglobin and *Limulus* hemocyanin are able to transfer the oxygen carried to mammalian tissue at the same velocity. No difference between the two pigments was found even though one contains iron and the other copper.

The experiments showed, however, that hemocyanin cannot be used for the perfusion of organs at the physiological temperature of 37° C. The perfusion liquid containing hemocyanin does not stand the temperatures above 25° C.; at higher temperatures precipitation of the pigment takes place and the perfusion comes to a standstill. For perfusion experiments of a relatively short duration, *e.g.* up to 10 hours, whole blood can be very well used. After that time, however, the perfusion liquid turns brown because of the formation of methemoglobin. The formation of methemoglobin in the perfusion pumps by the use of hemolyzed blood takes place at a similar rate. In trying to improve the oxygen carrying ability of the perfusion fluids used in the Carrel-Lindbergh apparatus, we studied the mechanism of methemoglobin formation in the absence and in the presence of living organs. The experiments were carried out with the Warburg technique as well as with the perfusion technique of Lindbergh and Carrel; the latter experiments were done in collaboration with R. Bing.

We studied first the influence of chain-breaking substances like pyrocatechol, hydroquinone and resorcine in suitable concentrations upon the formation of methemoglobin, because the chain character of this reaction has been suggested (2). Neither in hemolyzed blood alone nor in hemoglobin solutions in the Lindbergh perfusion pump in the presence of different organs was any influence of the chain-breakers on the formation of methemoglobin found. The fact that it seems rather difficult to prevent methemoglobin formation in perfusion experiments caused us to look for oxygen carrying pigments other than vertebrate hemoglobin. We hoped that erythrocruorin, the respiratory pigment of *Lumbricus terrestris,* would be suitable, because the large protein part of the molecule may conceivably stabilize the iron in the prosthetic group and render it more resistent to oxidation.

A detailed study of the physico-chemical and chemical properties of the pigment was therefore undertaken in collaboration with Kurt G. Stern. Erythrocruorin was prepared by repeated salting out or by repeated ultracentrifugation (67,000 g) of purified worm extracts. The ultracentrifugally prepared material showed only one sedimenting boundary in the analytical centrifuge.

Upon oxidation with potassium ferricyanide a band in the red appears, the center of which is at 645 mμ, 50 A towards the long wave region left

of the methemoglobin band. The band is less intense and less distinct than the corresponding methemoglobin band. Addition of fluoride at pH 5 shifts it to the yellow part of the spectrum without, however, producing an intensifying effect. We were not able to abolish the oxy-bands of erythrocruorin even with an excess of potassium ferricyanide. In general one may say that qualitatively the pigment is oxidized by the same reagents as hemoglobin; for instance, gallocyanin produces methemoglobin as well as meterythrocruorin in phosphate buffer at pH 7.5.

Erythrocruorin is not oxidized when oxygen is bubbled through its solution at room temperature for several hours. If, however, erythrocruorin is kept for 8-10 hours at 37° C., greenish brown particles of the denatured pigment precipitate. The precipitate is increased by shaking the solution of erythrocruorin several hours.

While erythrocruorine does not appear to be superior to hemoglobin for the purpose at hand, the study of this respiratory pigment has yielded some information about the properties of this chromoprotein. It has also demonstrated that the air-driven quantity ultracentrifuge is a suitable tool for the precipitation and purification of high molecular biopigments.

For a better understanding of the formation of methemoglobin *in vitro* it seems that further research is necessary to obtain more detailed information about the oxidation of hemoglobin by various reagents. The name methemoglobin has undoubtedly been applied to a group of substances of a somewhat different chemical constitution and therefore of different chemical reactivity. The study of these differences is the main problem of the oxidation of ferrohemoglobin.

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DISCUSSION

Dr. Hellerman: When methylene blue is added to mammalian erythrocytes, does the pace of the glycolytic process change?

Dr. Salomon: No; it is practically uninfluenced. The glycolysis may be a little enhanced, but not suppressed.

Dr. Hellerman: Do these cells contain flavoprotein? As I understand it, the action of the methylene blue upon the substrate is thought to involve a coenzyme system. Is that correct?

Dr. Salomon: I think so.

Dr. Hellerman: Without any undue emphasis upon the alleged scheme of an oxidation involving the utilization of methylene blue, I wonder what particular intermediate is postulated to oxidize the coenzyme on the basis of the Warburg scheme, if not flavoprotein.

Dr. Stern: I think Hellerman's point is well taken. When Warburg performed these experiments, the existence of flavoprotein was not known. The mechanism as shown by Salomon is perhaps a little over-simplified, and I am almost certain that actually a flavoprotein will oxidize the pyridine coenzyme, and that the reduced form of this yellow ferment will then be reoxidized by the methemoglobin.

Dr. Hellerman: There appears to be no evidence of the presence of flavoprotein in these cells, at present. I do not know whether a very painstaking analysis for flavoprotein has been made; but I should think that if it had been, we should have heard about it long before this.

Dr. Hisey: I think it may be of interest in this connection to mention some of the work of Wendel, who has shown that methylene blue is not a very effective agent for the formation of methemoglobin. On the contrary it will very rapidly reduce quite high concentrations of methemoglobin which have been formed by other methods. However, in the presence of cyanide, the accumulation of cyanmethemoglobin could be demonstrated.

Dr. Bernheim: Is anything known about the mechanism by which hydrogen peroxide produces methemoglobin? These dyes, when they autoxidize, produce hydrogen peroxide, which may play a part in the production of methemoglobin.

Dr. Salomon: It is true that leucodyes (*e.g.*, leuco methylene blue) upon reoxidation with molecular oxygen give rise to hydrogen peroxide formation. We are not too well informed on the fate of this hydrogen peroxide. While it cannot be denied that it might cause additional methemoglobin formation, as Bernheim suggests, it appears more probable that it will be destroyed as fast as it is formed by the powerful catalase present in red cells.

Dr. Bernheim: If you combine hemoglobin with various enzymes which produce hydrogen peroxide, methemoglobin is always formed. In the presence of cyanide you can get up to 70 p.c. of the theoretical yield; in the absence of cyanide you get 10-15 p.c. Even in the presence of a strong catalase you still get a certain amount formed from hemoglobin.

Dr. Warren: I cannot agree with one statement, and that is that the red cell is largely a glycolytic cell. I am speaking now of the mature orthochromatic red cell. It is perfectly true that in the Warburg apparatus one can measure the CO_2 formed from glycolysis a great deal more easily (because there is more of it) than the oxygen consumption. But when one calculates the energy available to the cell on the basis of the two processes, one finds that about 60 p.c. of the total energy available arises through respiratory processes, even though the respiration is very small.

In connection with the phenylhydrazine experiments, I think we ought to keep in mind the difference in behavior in intact tissues as compared with isolated systems. It has been shown for other cells, and I have seen it in the case of bone

marrow, that phenylhydrazine added to the cells, instead of increasing respiration, has apparently little effect on their respiration or anaerobic glycolysis; on the contrary, it appears almost exclusively to enhance aerobic glycolysis, in other words, to act as an inhibitor of the Pasteur reaction. This effect is over and above the effect which you spoke about with methemoglobin.

Dr. Salomon: Your point concerning the energy production in mammalian erythrocytes is well taken.

Regarding your second point, I would like to compare, as has been done by Warburg and collaborators, some of the properties of "phenylhydrazine"-cells formed *in vitro* with "phenylhydrazine"-cells formed *in vivo*.

effect of methylene blue on the respiration of mammalian erythrocytes certainly depends on the animal used. The stimulation of the respiration of the blood cells of the rabbit or man, for instance, is greater than that of horse or pig erythrocytes. The point I wish to bring out is the formation of catalytically different methemoglobins by different agents in the same species.

Dr. Hellerman: These different types are chemically different?

Dr. Salomon: I imagine they must be.

Dr. Barker: Is there any difference in the absorption spectra or other characteristics of these different compounds, whether they are catalytic or not?

Dr. Salomon: I do not think that anyone has

"Phenylhydrazine"-cells formed

	in vitro	*in vivo*
Color	brown	red
Respiration	stimulated	stimulated
Globin	denatured	denatured
Free hemin	present	absent
Addition of glucose	respiration stimulated	....
Carbon monoxide	inhibits respiration	inhibits respiration strongly
CO inhibition	light sensitive	not light sensitive
Glycolysis	....	10 $\times$
Oxygen transferred by	blood hemin	ferment hemin

As far as I am aware, the inhibition of the Pasteur effect by phenylhydrazine was first described by F. Dickens.

Dr. Shorr: In the formation and precipitation of methemoglobin from the preparation did you find any connection between the intensity of the respiration of the tissues and the rate of methemoglobin formation?

Dr. Salomon: Such experiments have not yet been done, so far as I know.

Dr. Baumberger: Did I understand correctly that when methemoglobin is formed by a biological system it has no catalytic action?

Dr. Salomon: I would not say quite that. When you produce methemoglobin *in vitro* you will find some methemoglobins which are catalytically active and others which are not.

Dr. Hellerman: Were they derived from different types of hemoglobin from different species?

Dr. Salomon: The order of magnitude of the

gone into the matter carefully yet. We investigated spectroscopically whether the addition of sodium fluoride acts in the same way on methemoglobins formed by different agents, but the fluor-methemoglobin bands produced did not show any difference.

Dr. Hellerman: In other words, these may involve chemical changes in the protein part and also in the porphyrin part?

Dr. Salomon: Yes. Mirsky and Anson have shown that the protein part and the porphyrin part of the hemoglobin molecule can be oxidized separately.

Dr. Stern: It might also be mentioned that the methemoglobin formed by ferricyanide is catalytically inactive. There is one instance, I think, of the formation of two different methemoglobins in human blood. Ordinarily when you add fluoride to methemoglobin the band in the red is shifted towards the yellow, but Keilin has in-

formed me that in a case of blackwater fever he examined a sample where the addition of fluoride, instead of shifting the band, produced a split in the band; this is interesting, because catalase shows exactly the same reaction when you add fluoride to catalase. But the pigment in question was not catalase, but a methemoglobin.

Dr. Warren: You mentioned that both methylene blue and methemoglobin can catalyze or stimulate the respiration of mammalian erythrocytes. Can you say anything about the chain of reactions through which those processes go? I believe there was considerable debate as to whether lactic acid was formed in the one system and not in the other.

Dr. Salomon: The respiratory quotient for the oxidation of hemoglobin by methylene blue, pyrrohemin and phenylhydroxylamine and others is below 1, mostly below 0.8. In many cases, the formation of pyruvic acid has been shown; in others, Warburg has simply made the assumption.

Dr. Velick: Your evidence that different methemoglobins are formed by different chemical agents is based almost entirely upon differences in the observed catalytic effects. Although one might certainly expect a series of reagents to produce different changes in the hemoglobin molecule, the nitrite, for example, producing a deamination, and the phenylhydrazine a reduction, the interpretation of the catalysis effect is rendered ambiguous by the fact that there are several other steps in the reaction chain which might also be affected differently or selectively by the reagents used. This seems especially likely if it is true that the reactions involve the protein portions of the catalysts.

RESPIRATORY CATALYSTS IN HEART MUSCLE*

Kurt G. Stern

When I first planned my address to this group, I intended to review the relation between respiratory ferments and cell structure, both on the basis of the data in the literature, and on the basis of recent work in our laboratory. I had included such topics as the chemical constitution of the respiratory ferment as inferred from photochemical and spectroscopic evidence, the effect of mechanical, thermal and chemical damage on cellular respiration, the relation of the Pasteur effect to cell structure, the influence of the nucleus on cellular respiration, and what happens to the respiration when egg cells are fertilized or divide.

All of these topics have, however, been ably discussed by preceding speakers in this Symposium. Instead of being repetitious, I propose to restrict myself to experimental data on respiratory catalysts in heart muscle that we have recently collected, and touch in a few instances upon the general implications of the results.

Historical

At the time when we began to interest ourselves in the respiratory catalysts in heart muscle Keilin and Hartree (1) had described a method for the preparation of cytochrome oxidase from heart muscle. They considered their final preparations to be fine suspensions of insoluble enzymes attached to heart muscle tissue. Besides these workers, Stotz and Hogness and their group (2, 3) had investigated the catalytic properties of such preparations. Both groups emphasized the essential role of cytochrome-c, which is largely lost during the preparation, and which must be re-added to make the system fully active towards substrates of the type of p-phenylene diamine and hydroquinone. It is now assumed that by measuring the rate of oxidation of hydroquinone or p-phenylene diamine under the conditions specified, a fair estimate of the cytochrome oxidase component of the respiratory chain of catalysts may be gained.

Earlier, J. Lehmann (4), in Thunberg's laboratory, and other workers, e.g. Stotz and Hastings (5), and Szent-Györgyi (6) had studied extensively the system capable of dehydrogenation of succinic acid to fumaric acid. Our own experiments in this field were begun for the following reason. M. K. Horwitt, chemist to the Elgin State Mental Institution, wanted to study certain enzymes in brain, and he came to our laboratory to acquire some experience in methods suitable for the study of such substances. When we discussed

possible attacks on his problem, succinic dehydrogenase was selected as a starting point, and since he planned to carry out the study of brain enzymes in his own laboratory I suggested we might investigate jointly succinic dehydrogenase in heart muscle.

Horwitt maintained at that time that several workers had been able to prepare succinic dehydrogenase in soluble form, and had also been able to transfer oxygen in aerobic experiments to succinate with the aid of such preparations. On the other hand, I believed that in all instances where I had seen reports on this enzyme, minced tissues, slices or tissue suspensions had been employed. It turned out upon a search of the literature, that when workers referred to succinic oxidase or succinic dehydrogenase preparations, and others spoke of cytochrome oxidase preparations, they all had reference to the same type of preparation. The two alternatives were either that the preparations represented mixtures of these two and other similar enzymes, or that there was some intrinsic structural relation between cytochrome oxidase and succinic dehydrogenase.

The first task in this work, therefore, was to find out whether succinic dehydrogenase can be classified as a soluble or as an insoluble enzyme. It will soon be evident that the question, stated in this way, is meaningless. Keilin had reported that his opalescent oxidase preparation upon filtering through filter candles invariably lost activity. He never reached the perfect zero level, but the decrease in activity upon repeated filtration was so great that there seemed to be little doubt but that he was dealing with a mechanical suspension. We proceeded to study the physical and chemical properties of the oxidase preparations obtained according to Keilin and Hartree. When heart tissue is minced in a meat grinder, and then ground with sand and washed, first a number of reddish and cloudy washing fluids are obtained until after the tenth or twelfth washing, when the wash water becomes colorless and clear. When at that stage the gray tissue pulp is treated with phosphate buffer at pH 7.3, an opalescent solution is obtained, which represents a crude but highly active oxidase preparation. The oxidase is precipitated by adding acetate buffer to bring the pH to 5 or 6. The precipitate is resuspended in phosphate, and the resulting suspension is the purified cytochrome oxidase preparation of Keilin and Hartree.

Succinic Dehydrogenase

The crude opalescent phosphate extract contains an active succinic dehydrogenase, as revealed by the rapid reduction of methylene blue in
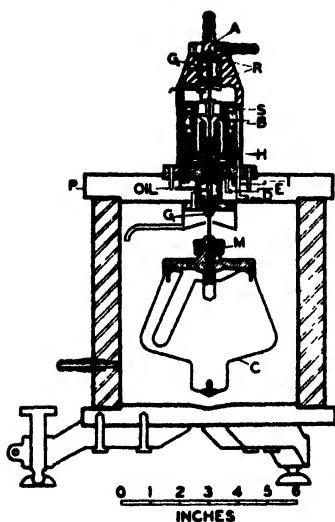
Fig. 1. Schematic longitudinal section through Beams air-driven ultracentrifuge (7).

a typical Thunberg experiment, with succinate as a substrate. Such active crude extracts were subjected to centrifugation in an air-driven quantity ultracentrifuge (Beams, *et al.,* 7). Fig. 1 is a schematical section through the type of ultracentrifuge used; the quantity rotor employed accommodates a total of about 100 cc. fluid in 14 celluloid tubes inclined in an angle of 25°. The rotor is first chilled in ice; during the run the temperature rises a few degrees, depending on the quality of the vacuum and the speed. In sedimentation experiments with this type of rotor, ordinary proteins which will not precipitate to form a solid pellet yield sharp boundaries. But if macromolecular material of the type present in our preparations is centrifuged, one finds pellet material at the end of the tube closest to the periphery; if the material is very heavy some of it will be deposited on the side wall of the tube. When the extract is spun for forty-five minutes at 250 revolutions per second, corresponding to a field of approximately 16,000 g, there is obtained a weakly opalescent supernatant liquid endowed with a small residual enzymatic activity. As a rule only liquids which are completely clear are inactive both with respect to *p*-phenylene diamine and to methylene blue after adding succinate. If the pellets found at the bottom of the tubes are suspended in phosphate buffer there is obtained a very active succinic dehydrogenase preparation. This first pellet solution was again spun for 120 minutes at 60 revolutions per second, which corresponds to a field of approximately 1,000 times gravity at the bottom of the tubes. Under these conditions the top layer in the tube is still appreciably active, but the bottom layer or the solid material is more active. Even at this low speed there occurs, therefore, a

definite sedimentation of the active principle. The supernatant fluids can be further fractionated; and, as a general result, one might say that the higher the speed the more completely the active principle is precipitated; at intermediate speeds (fields of 1,000 to 4,000 g and higher), some of the active principle is left in the supernatant fluid.

These observations confirm Keilin's statement that cytochrome oxidase (or succinic dehydrogenase) preparations represent suspensions of particles; and since they are sedimented by comparatively weak gravitational fields one can infer that they must have a rather large size.

The pellet solutions could be repeatedly sedimented. However, it was noted that the tighter the bottom fractions were packed the greater was the difficulty in resuspending them quantitatively and the greater the relative loss of activity. On the other hand, the greater the centrifugal force applied, the less activity remained in the supernatant solutions.

Cytochrome Oxidase

After Horwitt had left our laboratory, G. Scheff continued the work and examined the behavior of cytochrome oxidase under similar conditions. The following is a simplified account of one of his experiments: He started with the crude heart muscle extract which had a Q_{O_2} of 115 as determined against *p*-phenylene diamine in the presence of added cytochrome-c. This extract was divided into two parts: one part (a) was worked up according to Keilin and Hartree and the other part (b) subjected to the ultracentrifugal procedure outlined above. (a) The extract was brought to pH 6.1, the precipitate suspended in phosphate buffer, and the aggregated material was removed by centrifuging at low speed. The resulting opalescent solution, which corresponds to Keilin and Hartree's preparation, had a Q_{O_2}, when measured side by side with the crude extract, of 115. The agreement between the activity of the original and the purified preparation is fortuitous. (b) The other portion was ultracentrifuged for thirty minutes at 384 revolutions per second, developing a gravitational field of about 37,000 g. The pellets were suspended in phosphate buffer, and the aggregated material was removed in the laboratory centrifuge. The supernatant solution or suspension had a Q_{O_2} against *p*-phenylene diamine with added cytochrome of 215. It was, therefore, considerably more active per unit weight than the original extract. In this particular experiment the supernatant fluid obtained in the ultracentrifuge was not tested for its enzymatic activity.

It was interesting to see whether the oxidase preparations as prepared with the ultracentrifuge showed the same behavior towards the various substrates as the preparations obtained by isoelec-

tric precipitation. Fig. 2 and 3 show experiments which were performed with the aid of such centrifugal preparations. From the curves it may be seen that their behavior was the same as that of Keilin and Hartree's preparations. Against *p*-phenylene diamine the preparation is considerably active, and upon adding cytochrome there is a further increase in activity; but it is not as striking as in the case of hydroquinone.

There is a certain limit set to the purification, both by centrifuging and by isoelectric precipitation. At the outset it might appear that if the activity is tied up with the macromolecular material it would be preferable to employ sedimentation methods because if one shifts the pH one would expect to precipitate most of the proteins present in such preparations, since the isoelectric point of most tissue proteins is in the region around pH 4 to 5. By ultracentrifuging one may avoid the contamination of the macromolecular material with these "inert" tissue proteins. The difficulty in the case of the ultracentrifuge, however, is that, as I have already mentioned, the packing under high pressure seems to cause some irreversible change in the particles. And as one goes on with sedimenting and resedimenting, the bulk of the material finally fails to go back into solution, which can best be explained by assuming that irreversible aggregation has taken place.

Fig. 3. Catalysis of oxidation of hydroquinone by ultracentrifugally prepared heart muscle preparation:
Curve 1, oxidase preparation N + cytochrome-c;
Curve 2, oxidase preparation N without cytochrome.

In the case of Northrop's bacteriophage the activity is greatly diminished by ultracentrifuging once and abolished by centrifuging twice. In the case of the "smaller" plant virus proteins like the tobacco mosaic virus, the extent of irreversible aggregation under these conditions is small; and aggregation may be reversed by suitable procedures if it occurs. It was impossible to obtain fresh preparations which were active as cytochrome oxidase but inactive as succinic hydrogenase. Upon standing, however, the succinic dehydrogenase activity seems to decrease more quickly. Some aged preparations therefore showed an appreciable oxidase but no succinic dehydrogenase activity.

Spectroscopy

The spectroscopic observations made on purified oxidase preparations agree entirely with those reported by Keilin and Hartree (1, 8). Our preparations show strong absorption bands at 605 $m\mu$, particularly after adding a reducing agent such as $Na_2S_2O_4$, which is the α-band of cytochrome-a and at 565 $m\mu$, which is the α-band of cytochrome-b; there is also the α-band of cytochrome-c, at 550 $m\mu$, although relatively weakened, and a D band, at about 530 $m\mu$. We were also able to observe the disappearance of the band at 448 $m\mu$ and the

Fig. 2. Catalysis of oxidation of *p*-phenylene diamine by ultracentrifugally prepared heart muscle preparation:
Curve 1, oxidase preparation G + cytochrome-c;
Curve 2, oxidase preparation G without cytochrome;
Curve 3, cytochrome-c without oxidase.

strengthening of the band at 434 mμ after treatment with CO under the conditions recommended by Keilin and Hartree (8). This phenomenon is interpreted by these workers as due to the combination of the cytochrome-a$_3$ component (said to be identical with Warburg's respiratory ferment) with CO. We have never been able, by repeated centrifugation, to remove the cytochrome-c component completely from our preparations. The trend upon continued sedimentation is towards elimination of the -c and -b components and relative enrichment of the -a component.

Electrophoretic Separation Experiments

Since we were unable, with fresh preparations, to demonstrate any partition of oxidase and dehydrogenase activity upon centrifuging, we resorted to the electrophoresis apparatus of Tiselius (9).
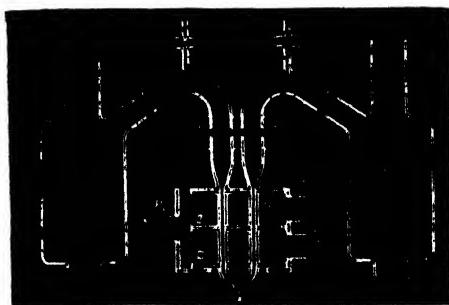
Fig. 4. Schematic longitudinal section through Tiselius electrophoresis cell for separation experiments (9).

Fig. 4 is a schematic drawing of the separation cell used. There are five compartments: left upper, right upper, left lower, right lower, and bottom cell. At the beginning of the experiment the enzyme solution is placed in the three lower compartments; the rest of the apparatus is filled with buffer; the cell is placed in a thermostat kept near 4° C. A suitable potential is applied through reversible Ag-AgCl electrodes and a mechanical compensation movement is started which increases greatly the resolving power of the arrangement. The progress of the electrophoresis is followed both by direct visual observations and by means of the Toepler *schlieren* method. At the termination of the experiment the various compartments are shut off from each other with the aid of pneumatic pumps, the apparatus is dismantled, and the various fractions are taken out and assayed. The Tiselius arrangement used in our laboratory is described in detail elsewhere (10, 11).

The results of such an experiment are shown in Table I.

Another experiment of this type gave a similar result. Neither experiment indicated a clear partition of the activities among various protein carriers; the ratio of the enzymatic activities in

TABLE I.

Electrophoretic separation experiment with heart muscle oxidase preparation K$_2$. 0.02 M phosphate buffer, pH 7.6; 6° C.; current varied from 180 v., 5.6 ma. to 270 v., 8.2 ma. Rate of compensation movement, 30 min., 1 hr. Duration of run, 200 min.

Compartment	$Q^P_{O_2}$*	Q^S_{MB}**
Anode limb:		
upper cell	15.7	≈0
lower cell	67	15
Cathode limb:		
upper cell	84	4.4
lower cell	75	33
Original preparation:	80	33

* Uptake of O$_2$ in μl. per hour per mg. dry weight of preparation with *p*-phenylene diamine as substrate and an excess of cytochrome-*c*.
** Anaerobic activity towards succinate with methylene blue as acceptor, expressed in terms of "μl." methylene blue reduced per hour per mg. dry weight of preparation. In calculating these values, the initial rate of methylene blue reduction, rather than the total reduction time, was used. The former was deduced from the latter under the assumption that the process is of the first order with respect to both methylene blue and enzyme concentration.

the various fractions was not the same throughout, but the accuracy of the tests is not very great and differential inactivation may have occurred. In no instance was it observed that the cytochrome oxidase migrated to one side and the succinic dehydrogenase to the other.

Experiments with the Analytical Ultracentrifuge

The results of our fractionation experiments led to the adoption of the working hypothesis that the various enzymatic activities which are present in the heart muscle preparations may not be due to a large number of different protein bearers, but that perhaps one is dealing here with a system where a number of active groups are attached to one common carrier. We therefore proceeded to examine these preparations in an analytical air-driven ultracentrifuge. We used Svedberg's light absorption method throughout; the Toepler *schlieren* method is not suitable for turbid systems. We worked both with blue light, at which these preparations show definite absorption, and also with ultraviolet light, using a bromine filter and leaving out the chlorine filter, so that a broad wave band is produced which is much less affected by the true protein absorption in the short wave ultraviolet region and is therefore

Fig. 5. Series of photographs obtained during ultra-centrifugal sedimentation of heart muscle oxidase preparation N: 9 mg. of three times ultracentrifugally purified oxidase preparation in 1 cc. 0.1 M phosphate buffer, pH 7.3; mean gravitational force, 3500 g. (7200 r.p.m.); interval between exposures, 5 min.; exposure time, 1 sec. (Eastman positive-film); λ = 2480 — 3600 A (high-pressure mercury vapor lamp, bromine filter); maximum temperature during run, 30°; magnification factor of centrifuge camera, 1.5.

mainly affected by the light scattering of the large particles present in the solutions.

Fig. 5 is a series of photographs obtained from an oxidase preparation during a centrifuge run.

We have never encountered preparations which showed more than one boundary. The general phenomenon observed in these experiments is that while the boundary at first is reasonably sharp, less than half way through the sector-shaped cell the boundary begins to shade off, and we have never been able to follow the sedimentation down to the bottom of the cell. It is a sort of "disintegration process", which we have observed also in the case of other macromolecular substances.

When these photographs are subjected to analysis by a microphotometer, records of the type shown in Fig. 6 are obtained. All of these curves show the familiar S-shape; however, they tend to change their slope, and finally the curve representing the last exposure is almost lying on its back, indicating that we are dealing here with particles of overlapping sizes. In contrast to

random suspensions, however, there exists a certain average particle size which can be measured.

A three-times ultracentrifuged, fresh preparation gave sedimentation constants of 283 and 295 $\times 10^{-13}$ cm. sec.$^{-1}$ dyne^{-1} as calculated from the microphotometer curves. The application of Stokes' equation leads to a diameter of 50 millimicrons, and a molecular weight, if we can call these particles molecules, of 47 million. With one of our earliest preparations we obtained a sedimentation constant of 770, corresponding to a diameter of 80 mμ and an apparent molecular weight of 202 million. Three other preparations gave sedimentation constants corresponding to diameters of 178, 192 and 196 mμ and to weights of the order of 2 billion.

This is only a progress report and much more work will be necessary to establish the details with certainty. It is not impossible that the large particles arise through aggregation of the smaller ones.

Shape of Particles

Why do we feel justified in applying Stokes' equation to a calculation of particle size from these sedimentation constants? First, because microscopic examination in the dark field of these and very similar animal virus preparations, like Rous chicken sarcoma agent, shows a multitude of round particles which are actively engaged in Brownian movement. The viscosity of the preparations is rather high in 1 p.c. solutions (1.2); it is higher than one might expect from perfectly spherically shaped particles. However, while it is true that strongly asymmetrical particles always show a high relative viscosity, one cannot safely reverse the statement and say that because a particle gives a somewhat viscous solution it must be asymmetrical.

Furthermore, we have examined our fractions at various stages of the preparation with the polarizing apparatus of Takahashi and Rawlins and that of Ferry. I have sent one of our preparations to Lauffer in Stanley's laboratory and he was unable to discover the slightest amount of double refraction of flow under conditions where it could readily be detected in tobacco mosaic protein solutions. This being so, we felt justified, for the time being, in applying Stokes' equation for spherical particles. It is hoped that at a later time the exact shape of these particles will be amenable to study with the electron microscope.

This also throws an interesting side-light on Keilin's statements. He considers his oxidase preparations as fine suspensions of muscle tissue. Now slices of muscle tissue show a beautiful phenomenon of intrinsic birefringence. The same is true for fine mechanical suspensions of muscle tissue. Preparations of muscle proteins contain-
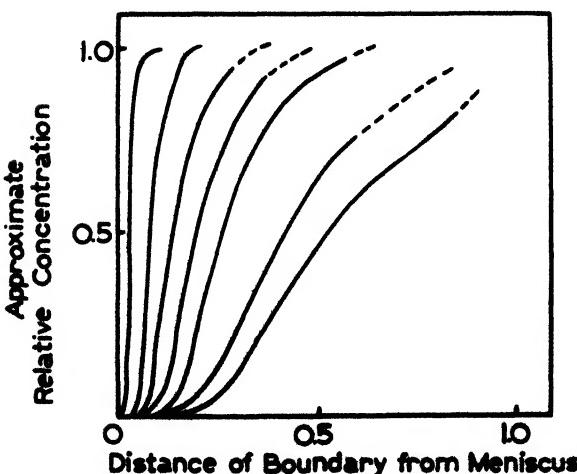

Fig. 6. Tracings of microphotometer curves obtained from the 1st, 2nd, 3rd, 4th, 5th, 7th and 8th exposure of the photographic series shown in Fig. 5. Recording magnification ratio 1:6.

ing myosin will exhibit strong flow birefringence.

Since our particles show neither intrinsic double refraction nor double refraction of flow, I think that this disposes of Keilin's statement that he was dealing with muscle suspensions. This is no contradiction to Hopkins' (12) observation that exhaustively extracted minced skeletal muscle still contains some dehydrogenase activity and exhibits birefringence. The first NaCl extracts were found to contain colloidal particles and also dehydrogenase activity. The birefringence of the insoluble residue is not necessarily due to the oxidase contained in it.

There is another point which makes one wonder whether a distinction between soluble and insoluble respiratory catalysts has any meaning. If these oxidase preparations are really only mechanical suspensions of particles of heart muscle tissue, there is no reason why one should not obtain them by tearing up the tissue in the preliminary processes in the meat grinder, or by grinding with sand and subsequent treatment with water. The Rous chicken sarcoma agent, incidentally, will readily go into distilled water if the tissue is treated under the same conditions; but not so the oxidase. When one refrains from any further mechanical treatment, just putting the ground and washed tissue in distilled water, the filtrate is colorless and clear. But if phosphate buffer is now added, an opalescent and active solution is obtained. Since there is no mechanical treatment involved, this observation already suggests that we are not dealing with a mechanical suspension of muscle tissue, but that a macromolecular component has been extracted by solvent action.

Chemical Composition.

We have also done some work on the chemical properties of these preparations and it has turned out, interestingly enough, that in chemical composition they are almost indistinguishable from certain animal virus preparations. For instance, J. L. Melnick finds the nitrogen content of muscle oxidase preparations by the micro Kjeldahl method to be 9 p.c., about the same as that of the purified Rous virus (cf. 13) and that of the chicken leucemia material (14). All three preparations contain hemin, as shown by the pyridine hemochromogen test; all preparations contained nucleic acid. Claude has found that the residual matter in his Rous virus preparations is lipoid and we have little reason to suspect that it should be different in our case. The cytochrome oxidase activity is very high in the heart muscle preparations and, as Melnick finds, is significant, but not as high in the purified Rous and leucemia virus preparations. Catalase is present in all three. In fact all these preparations in their composition resemble protoplasm rather than pure proteins of the type of the tobacco mosaic nucleoprotein of Stanley.

Nature and Significance of Oxidase Particles.

We are dealing with a complex system. What is the nature of these particles, and how quantitatively significant is the activity of such preparations as compared with the respiratory activity of intact heart muscle? Unfortunately experiments with intact tissue slices and p-phenylene diamine have not been reported as yet.[*] All I can do at present is to compare the oxygen uptake of heart muscle slices without external substrate with the power of oxidase preparations to transmit oxygen to p-phenylene diamine and hydroquinone. Shorr was kind enough to supply me with data concerning intact heart muscle. He informs me that the Q_{O_2} of the heart muscle tissue of the dog and cat, in the absence of external substrate, is between 2.5 and 3.5. (This refers to dry weight of heart muscle.) I have calculated the quantitative relationship for some of our preparations. Take, for example, the three-times ultracentrifuged preparation M. The total amount of the washed and ground heart muscle used in this preparation, assuming $Q_{O_2} = 3$, had an oxygen uptake of 138,000 μl. per hour. The purified preparation itself, when tested with hydroquinone and added cytochrome-c, had a Q_{O_2} value of 230. This yields for the total preparation a value of 33,100 μl. per hour. In this experiment no attempt whatsoever was made to make the extraction quantitative. It is probable that much of the oxidase activity remained in the pulp under our conditions of extraction. In spite of this as much as 24 p.c. of the total respiratory activity of the tissue can be accounted for by the cytochrome oxidase activity present in the purified extracts.

The same calculation for the three-times ultracentrifuged preparation O leads to a percentage of 39 p.c. oxidase recovery.

We also calculated this relationship for a crude heart muscle extract. This extract was prepared from tissue which had a total respiratory activity of about 300,000 μl. per hour. The crude extract, when tested for cytochrome oxidase, had an O_2-uptake of 631,000 μl. per hour. In other words, the activity present in this case in the crude extract could account for double the amount of normal cell respiration which is exhibited by this tissue. Although I do not wish to attach too much weight to the actual figures, it is obvious that we are not dealing with an insignificant fraction of the oxidase activity of the heart muscle tissue. The recovery in the case of the ultracentrifuge preparations appears surprisingly high.

[*] The paper by Elliott et al. (Biochem. J. 32, 1407, 1938) contains oxidase measurements on various tissues after "homogenization".

I believe that these preparations do not represent suspensions or mixtures of entirely heterogeneous material. I believe, on the contrary, that it is no accident that the physical and chemical properties of these oxidase preparations should so closely resemble the properties of the various animal virus proteins which we and other workers have studied recently. The explanation which might be advanced, that one is dealing with a bulk of inactive material, and that in one case the virus activity and in the other case the oxidase activity is due to an unknown agent which is present in traces has as little probability value as it has in the instance of the crystalline proteins showing enzymatic or virus activity.

Upon searching the literature, I found a paper interesting both for its subject and for its author. The author is Otto Warburg, who so often is quoted as having intrenched himself in the idea that the respiration of the cell and the respiratory enzyme are insolubly anchored to the architecture of the cell. When Warburg speaks of structure he does not mean cell architecture. As a matter of fact, I think that Warburg in 1913, when he published the paper entitled "Uber sauerstoffatmende Körnchen aus Leberzellen" (15) has made, perhaps in a more qualitative manner, observations which completely agree with the conclusions we draw from our experiments.

Upon crushing liver tissue, suspending it in saline and centrifuging the mixture for a brief period, Warburg obtained a sediment consisting of cells and cell debris and a supernatant suspension containing fine granula which he described as "kleine, runde, in Brown'scher Molekularbewegung begriffene Gebilde". He considers these small particles, which exhibit an intense respiration, as probably identical with the preformed cell granula. Thus blood cells, which contain but few preformed granula, yield extracts containing almost no granula in the supernatant solution and showing no respiration. Centrifugation of liver extracts for one hour failed to yield preparations free from granula and devoid of respiratory activity. However, upon filtration of the suspensions through a Berkefeld candle, solutions free from visible granula and exhibiting a small but definite respiration were obtained. The active fractions were opalescent in incident light and almost clear in transmitted light. Under the microscope, "sieht man im Hellfelde ein ganz feines Wimmeln, aehnlich wie im Serum, waehrend die Koernchen verschwunden sind".

I think that we should stop quoting Warburg as postulating that it would be impossible to extract the respiratory units from cells, because the word "structure", which he has used, is so often confused with the words "intact cell architecture".

Microscopical granula endowed with respiratory activity have been described and studied by a number of subsequent investigators. It may suffice here to mention the recent work of Banga (16) in Szent-Györgyi's laboratory and of Greville (17) on dispersions prepared from pigeon breast muscle. These suspensions, obtained by treating the finely minced tissue with cold distilled water, contain particles so small as to pass through Jena G.3 glass sintered filters and to be difficult to centrifuge, except in an angle centrifuge after dilution with water (Greville, 17). According to Banga (16), the sediment obtained contains "enzymes" and the supernatant solution contains an "activator", both being essential to the respiratory activity towards carbohydrate. Szent-Györgyi (6) pictures these granula as anchoring bases of succinic and malic dehydrogenase, cytochromes, and of cytochrome oxidase, i.e. of the catalytic system required, according to his theory, for the terminal oxidation of metabolites. Now it appears that pigeon breast muscle is an exceptional material in various respects, not only with regard to the ease with which it may be dispersed in distilled water. The particles of Banga and Szent-Györgyi cannot, therefore, be called typical for those obtainable from the tissues of other higher animals. But there is little doubt that they have essential features in common with the smaller particles constituting the beef heart muscle oxidase preparations which we have studied. We consider our particles as "sub-cellular" functional units and we believe that the active groups of the various component catalysts are arranged in or on them in an orderly fashion so as to ensure a smooth functioning of the highly complex process of cellular respiration. This does not mean that these functional units must represent chemical individuals in the usual sense of the term. On the contrary, it is quite possible that further work will result in the dissection of these units into the contributing entities. The recent experiments of Hopkins et al. (18) on the isolation of the succinic dehydrogenase from muscle are perhaps a first step in that direction.

Mechanism of Succinate Oxidation*

There is one more point which I would like to discuss briefly, namely, the question of the mechanism of succinate oxidation in heart muscle. When we began this work it seemed generally accepted that the succinic dehydrogenase system is directly linked to the cytochrome system. This is an important point in Szent-Györgyi's theory (cf. 6). Since we had no reason to doubt the validity of this concept, we accepted it at its face value; and in the earlier stages of our work we tested for succinic dehydrogenase by the classical

* See also Stern and Melnick (19).

methylene blue technique of Thunberg and for cytochrome oxidase by the manometric technique. Recently I suggested to Melnick that he place a heart muscle preparation in a Warburg manometer and add succinate as a substrate to see whether it was as actively oxidized as was *p*-phenylene diamine or hydroquinone. To our surprise the purified preparation was completely unable to transfer oxygen to succinate under these conditions. We now went back and examined the aerobic and anaerobic activities of such preparations to succinate in the course of purification. Cytochrome-c could not be the missing link, but when a small amount of the supernatant liquid from the ultracentrifuge run was added the aerobic activity towards succinate immediately rose to the original level of the crude extract. Fig. 7 represents a typical experiment. It was found that the degree of activation was roughly proportional to the amount of supernatant liquid added. There is then a supplementary principle present in these extracts which is removed in the course of centrifugation, and the removal of which renders the purified preparations unable to transfer oxygen through the respiratory ferment-cytochrome system to the succinic dehydrogenase system. This principle is not identical with aluminum (20) and not with catalase (21). However, when these solutions are subjected to high-speed centrifugation at 500 revolutions per second for two hours, corresponding to a maximum field of 67,000 times gravity, the activator is partially sedimented.

A particle behaving in this way may be expected to have a molecular weight of about 140,000. Thermal inactivation and precipitation tests with trichloroacetic acid suggest that this principle is a protein. Its possible relationship to Straub's flavoprotein from heart muscle (22) is under investigation.* The partial or total loss of aerobic activity towards succinate observed by Stotz and Hastings (5) and by Hopkins (18) may have been due to the removal of this principle.

The existence of such a "soluble" factor would have to be postulated on theoretical grounds if the principle of interaction of "stationary" and diffusible catalysts developed by Szent-Györgyi, Green and others is brought to a logical conclusion:

oxygen → {respiratory ferment} × {cytochrome-a} *cytochrome-c* {cytochrome-b} × {succinic dehydrogenase} *diaphorase* ↔ pyridine enzymes ↔ *coenzymes* ↔ substrates.
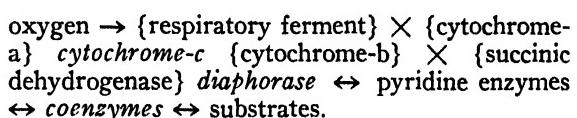
In this scheme the "stationary" catalysts are bracketed. At the places marked by × coupling by a "diffusible" carrier would be expected.

Fig. 7. Catalysis of aerobic oxidation of succinate by heart muscle oxidase preparations:

    Curve 1, unfractionated heart muscle extract + cytochrome-c;

    Curve 2, ultracentrifugally purified oxidase preparation N + supernatant fluid from ultracentrifuge run + cytochrome-c;

    Curve 3, oxidase preparation N + cytochrome-c;

    Curve 4, oxidase preparation N without cytochrome-c.

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DISCUSSION

Dr. Bernheim: We once tried to purify the succinoxidase of liver by washing and centrifuging with an ordinary centrifuge and keeping the pH on the acid side. After a long procedure we finally got a preparation which was quite active and when looked at under a microscope after staining with methylene blue consisted practically entirely of nuclei. I wonder whether the qualitative observation, plus the fact that you have nucleic acid in your preparation, might not suggest that the succinoxidase is associated with the nuclei?

Dr. Stern: It is, of course, possible that the cell nucleus is a carrier of at least a part of the succinic oxidase activity of the cell. Furthermore, if by your procedure you have succeeded in isolating nuclei in enzymatically active state you have made an important contribution to the problem of nuclear function in general. The nature of our data does not permit a conclusion as to the origin within the cell of our macromolecular material which is endowed with the various catalytic properties described here. The smallest active particles which we have encountered in our work would appear to be of an order of magnitude below that commonly assigned to cell nuclei. But that does not exclude their having been derived from nuclei by a disintegration process.

Dr. Lipmann: What do you think about the hydrogen peroxide formation which Lehmann has found during succinic acid dehydrogenation? It seems to point to a flavin intermediate.

Dr. Stern: I admit that Lehmann's observations seem to suggest the participation of a flavoprotein in the catalysis. On the other hand, we have no evidence at present which would indicate that our activating principle is a flavoprotein.

It is not possible, at present, to determine the exact position of the supplementary principle in the respiratory chain of catalysts. If it were the coupling link between succinic dehydrogenase and the cytochrome system, one would expect to find ultracentrifuged oxidase preparations incapable of reducing ferricytochrome. However, Melnick, working in my laboratory, observed that such purified preparations which, in the absence of the activator, were unable to transfer oxygen to succinic acid, were still able to catalyze the reduction of cytochrome when examined spectroscopically. The question will probably be decided by quantitative, rather than qualitative, experiments. It may be that the rate at which such preparations can reduce cytochrome is insufficient to produce an appreciable oxygen uptake under the conditions of the manometric experiments.

Mr. MacLeod: Bernheim's questions about the nucleus and its relation to cellular metabolism as a whole raises a fundamental question. The mammalian red cell is a case in point. Warren has shown that the O_2 consumption of the reticulocyte is about 30 times that of the mature red cell, and Kempner has shown that the erythroblast has a Q_{O_2} of 16, a relatively enormous respiration compared to that of the mature red cell. In these two cases, the increasing respiration is coincident with increasing nuclear content, since the reticular material of the reticulocyte is generally considered to be the remains of the normoblast nucleus. Furthermore, Ponder and I found that the nuclei of neutrophils released from rabbit bone marrow after stimulation with nucleic acid showed a different staining reaction from those liberated under normal conditions. The nuclei also were much larger than those of cells liberated normally. These "nucleic acid" cells have a respiration about twice that of normal cells. In all of these cases there would seem to be a definite relation between the state of the nucleus and the respiration of the cell.

Do you know of any figures on the succinic dehydrogenase or cytochrome oxidase content of reticulocytes?

Dr. Stern: No. Although the observations of Warburg on the negligible respiratory activity of non-nucleated mammalian red cells as compared with the considerable oxygen consumption of nucleated avian erythrocytes are very suggestive, there does not seem to exist conclusive evidence to show that the respiratory activity of cells resides in their nucleus or that the presence of the nucleus is always a necessary prerequisite for the manifestation of cellular respiration.

Dr. Velick: It is a very convenient explanation of the high respiration of the reticulocyte to say that the reticular substance is nucleus in the process of degeneration. This seems to be pretty definitely not the case, and I think there is general agreement that the reticular substance is not nuclear material.

Mr. MacLeod: I have not yet seen any clear proof that the reticular material of mammalian reticulocytes is not the remains of some nuclear material. The fact that this material does not stain with ordinary nuclear stains is not proof that it was not at one time part of a nucleus. It does

not follow that the remains of a nucleus in the process of being disintegrated should necessarily show the same chemical reaction as that of the intact nucleus. However, the fact remains that some chromatic material within the mammalian reticulocyte is associated with the relatively high respiration of that cell.

Dr. Barker: Is it possible that a high oxygen consumption of the oxidase preparation as compared to that of the original heart tissue might be due to the experimental conditions? In measuring oxidase activity you logically fix the conditions so that the oxidase activity is the limiting factor, whereas this may not be so in the intact cell. That would also enter into the comparison Stotz tried to draw when he compared the cytochrome-c and oxidase contents of various tissues with the Q_{O_2}. The heart muscle cytochrome and oxidase content was very high, although its respiration was lower than that of other tissues having less cytochrome. Perhaps something else is limiting the oxidation chain.

At the same time, I think it is very interesting that the oxygen consumed by these preparations is roughly of the same order as that used by the whole heart tissue, instead of being several times greater. This would indicate that normally one is constantly obtaining a rather complete utilization of the cytochrome oxidase system.

Another thing which seems striking is the cytochrome oxidase activity of the virus preparations. Has that been reported before? Are there any other enzymatic activities of the various preparations?

Dr. Stern: It still remains to be shown that the cytochrome oxidase and catalase activity of purified chicken sarcoma and fowl leucosis virus preparations is an intrinsic property of the active agent, and not due to associated substances. However, it should be mentioned that there are other observations on record concerning the manifestation of enzymatic activity by virus material in a few isolated instances. Thus, Schueler in Meyerhof's laboratory has reported the presence of phosphatase activity in a purified preparation of a *B. coli* bacteriophage. On the other hand, all attempts to demonstrate a metabolic activity, such as glycolysis or respiration, of purified and concentrated bacteriophage or vaccinia elementary body preparations have thus far been negative. This does not exclude the possibility that further work may demonstrate the ability of virus materials to utilize certain intermediate metabolites. *e.g.* succinic acid, in energy-yielding reactions. It would appear that the definition of "metabolic activity", if based on the utilization of glucose or lactate only, is somewhat arbitrary and may require some modification in the future. A virus particle may have lost, in accordance with the views expressed by Laidlaw in his recent Reede

Lecture, its biochemical independence in the course of prolonged parasitic existence. But this loss may have affected only the ability of the parasite to handle substrates of a higher or more complex order, *e.g.* glycogen or hexoses; it may still be able to burn simple substrates such as succinate and to utilize the resulting gain in free energy for the synthesis of virus protein. In general, I am inclined to agree with the view of Holmes and Pirie in their contribution to the volume "Perspectives in Biochemistry" that the case against the nature of pathogenic animal viruses as small organisms rests on inadequate evidence.

Dr. Baumberger: Your work seems to point to the molecular complex having the function of coordination of the oxidase with the dehydrogenase through the series of cytochromes. In your preparation, cytochrome-c diffused out from its absorbed condition in the molecular complex relatively easily. It may be that the activator you find reinserts the cytochrome-c in its proper position. It seems to me that the observations I have made on cytochromes in intact cells fit in somewhat with the picture I have drawn from your presentation in that I am unable to get cytochrome-a reduced unless I have cytochromes-b and -c also reduced. When an oxygen molecule plus oxidase act on this series, all three cytochromes are oxidized. When activated hydrogen is added all three molecules are reduced. When I speak of all three, I mean they may be parts of the coordinated molecular complex you suggest.

Dr. Melnick: That a system containing cytochrome oxidase, cytochromes-a, -b and -c, and succinic dehydrogenase fails to oxidase succinate aerobically may indicate, as Baumberger suggests, that a factor is missing, the function of which is to line up the respective components in their proper positions in the respiratory chain. However, cytochrome-c is reduced in the presence of succinate by the ultracentrifugally prepared oxidase. This indicates that the reduced succinic dehydrogenase is still capable of reacting with cytochrome. The catalytic effect of cytochrome-c in the oxidation of hydroquinone by the oxidase preparations demonstrates that reduced cytochrome-c is readily oxidized by the enzyme preparation. As the activating principle is inhibited by cyanide, it is probable that it plays a role in linking the dehydrogenase and cytochrome-cytochrome oxidase systems. The explanation of the cytochrome reduction by the preparation in the presence of succinate may lie in the rate of the reduction, which may turn out to be too slow to procure an appreciable oxygen uptake.

Dr. Graubard: Is the activator insoluble in water?

Dr. Stern: It is soluble in water.

Dr. Graubard: Then how is it that most of

the succinic dehydrogenase preparations have no activator after ten or twenty washings with water, and nevertheless it remains with the solid material of the heart tissue?

Dr. Stern: The activating principle seems to be present in great excess in heart tissue. Nevertheless, Stotz and Hastings have already observed that upon extended washing, heart muscle preparations begin to show decreased activity towards *p*-phenylene diamine and hydroquinone even after addition of cytochrome-c. They speak of an "incomplete enzyme system" in their report. This observation can be explained satisfactorily by the gradual removal of the activating principle by the procedure they used. That there is still some activating principle left in the preparation is due to the inferior solvent action of water or saline as compared with 0.1 M phosphate buffer.

Dr. Melnick: The advantage of ultracentrifugation over isoelectric precipitation in the preparation of macromolecular material may be seen in a comparison of the nitrogen contents of such preparations. The ultracentrifugally prepared oxidase contains about 9 p.c. N, whereas the isoelectrically precipitated preparations contain 11.5 p.c. N. The higher nitrogen content suggests that inert tissue proteins are also precipitated from the heart muscle extract upon lowering the pH from 7.2 to 5.5. These proteins are left behind in the supernatant fluid upon ultracentrifugation.

It should also be mentioned that the oxidase preparations made according to the method of Keilin and Hartree, *i.e.* by isoelectric precipitation, contain succinic dehydrogenase, cytochrome oxidase, cytochromes-a and -b, and small amounts of soluble cytochrome-c; in the oxidation tests cytochrome-c must be added for maximal activity. Since succinate, as well as hydroquinone and *p*-phenylene diamine, is oxidized by the preparation, the activating principle must also be present. However, by twice repeating the isoelectric precipitation, the aerobic activity towards succinate is greatly impaired whereas that towards hydroquinone remains unaltered; it appears that such treatment, too, removes the soluble activator. If the activating principle, present in the supernatant fluid when the oxidase is sedimented in the ultracentrifuge, is added to a three-times isoelectrically precipitated oxidase, the aerobic activity towards succinate is restored.

Dr. Lipton: If one adds reduced cytochrome-c to that system, is the supernatant fluid required only to reoxidize the reduced cytochrome-c?

Dr. Melnick: The oxidase preparations, in the absence of the activating principle, are capable of readily oxidizing reduced cytochrome-c. This is indicated by the catalytic effect of cytochrome-c in the oxidation of hydroquinone by the preparations.

Dr. Lipton: What I am trying to do is to locate the position of the activator contained in the supernatant fluid.

Dr. Stern: Speculations on this point would seem to be premature. All that one can say at present is that a soluble principle of protein nature is required for the rapid aerobic oxidation of succinate by a heart muscle oxidase system containing cytochrome oxidase, cytochrome and succinic dehydrogenase.

THE RELATION OF HORMONES TO CARBOHYDRATE METABOLISM *IN VITRO**

Ephraim Shorr

The nature of the mechanisms governing carbohydrate metabolism has long been of major interest to workers in the field of intermediary metabolism. That it is under the control of more than one hormonal factor is now a matter of universal agreement. Prior to the fundamental work of Houssay in 1929, insulin was regarded as the only hormone essential for the metabolism of carbohydrate and attention was focussed entirely on experiments designed to ascertain the mode of action of this hormone. Recognition that the condition was more complex followed Houssay's demonstration that the secretions of the anterior pituitary, functioning as antagonists to insulin, were also involved. Later, Long and Lukens (1) called attention to a similar inhibitory role of the adrenal cortex. The older concept was supplanted by one which postulated a system regulating carbohydrate metabolism made up of an accelerating component, insulin, and inhibitory factors elaborated by the pituitary and the adrenals. The failure of carbohydrate metabolism in diabetes resulted from an unchecked action of these inhibitory influences in the absence of insulin.

While this new concept represented a significant advance, it has not as yet been productive of any more exact information regarding the mechanisms by which either of these two groups of hormones produces its effects. The data obtained on the whole animal have been concerned with such overall phenomena as oxidation and storage of carbohydrate. The non-specific character of this type of information has favored the use of *in vitro* methods as more likely to throw light on the set of reactions by which carbohydrate is utilized in the cell and the exact manner in which insulin and its antagonists enter into these reactions.

However, the results of the search for *in vitro* effects of insulin have been disappointing. Very few of the many insulin effects reported have survived careful scrutiny. A few of the more recent are provocative, and may be mentioned briefly. Working with excised liver slices, Bach and Holmes (2) confirmed the *in vitro* synthesis of carbohydrate by this tissue previously reported by Gemmill and Holmes (3). They noted in addition that insulin reduced the extent of gluconeogenesis. This diminution was accompanied by a depression of urea formation, not only under basal conditions, but also in the presence of glycogenic amino acids. Gluconeogenesis from lactate and pyruvate was uninfluenced by insulin. These workers concluded

that insulin prevented gluconeogenesis in liver slices by exerting a protein-sparing action, an interpretation in accord with one of the actions of insulin in diabetes. However, the results of Stadie, Lukens and Zapp (4) were not in full agreement with those of Bach and Holmes in that the depression of urea formation by insulin was not confined to glycogenic amino acids and was most consistent with the non-natural forms. Furthermore, they failed to find any effect of insulin on carbohydrate synthesis by liver slices.

Seckel (5), working with the same tissue and determining glycogen alone, observed a sparing effect of insulin on glycogenolysis. His observations, while not strictly comparable to those of Bach and Holmes, who measured total fermentable carbohydrates, would seem to be at variance with the direction of the carbohydrate change found with insulin by these workers. More work will be necessary before these observations can be accepted as indicating the mode of action of insulin on the liver.

An insulin effect on muscle metabolism has recently been reported by Krebs and Eggleston (6). Employing pigeon breast mince and a medium which contained phosphate, boiled muscle juice and citrate, they observed a marked increase in the respiratory rate in the presence of insulin. In addition to being the first *in vitro* demonstration of an insulin effect on oxidative metabolism, these experiments gain further interest because they point out a possible mode of action of insulin, namely, as a catalyst of the postulated citric acid cycle. These experiments of Krebs and Eggleston will be discussed at length later in this paper.

The reasons for the many failures to obtain significant *in vitro* insulin effects are obscure. It is conceivable that the reaction to insulin is complex and dependent on the participation of several tissues in the body. However, the demonstration by Cruikshank and Startup (7), that the diabetic heart-lung preparation was able to oxidize carbohydrate when insulin was added to the perfusion fluid, is evidence against this concept. The possibility of permeability difficulties *in vitro* has also been suggested as a reason for the negative results. Krebs has suggested as an explanation for the insulin effect on the pigeon mince, the ability of insulin to reach the reactive systems as a result of cellular disintegration.

Whatever the reasons, more specific knowledge of the intermediary metabolism of tissues under normal and pathological conditions should facilitate progress on this problem by affording criteria for evaluating any possible insulin effects. For

some time in this laboratory[1] experiments have been carried out on the comparative respiratory metabolism of tissues derived from animals in the normal state and after the removal of the pancreas or pituitary, or both. Attention was focussed on the oxidative and glycolytic mechanisms to ascertain which aspect was primarily influenced by insulin. In the course of these experiments it became necessary to reexamine the current concepts of the Pasteur reaction, particularly that proposed by Meyerhof.

The results of these experiments cast doubt on the validity of the concept of the Pasteur reaction which regards lactic acid as a necessary intermediary for the oxidation of carbohydrate in animal tissues. They also indicate that energy for resynthesis of lactic acid may be derived not only from the oxidation of lactic acid, or equivalent carbohydrate, but of other foodstuffs as well. Our data also suggest that the primary defect in carbohydrate metabolism in the absence of insulin resides in the oxidative mechanism and not in the lactic acid enzyme system, inasmuch as glycolysis and lactic acid resynthesis can proceed normally in diabetic tissue unable to oxidize glucose. Removal of the pituitary from the diabetic dog resulted in a restoration of an apparently normal carbohydrate metabolism *in vitro* to tissues from these animals. These observations support the view that the carbohydrate metabolism of the cell is fundamentally independent, and may go on without the intervention of hormonal influences which have a regulatory function made necessary by the increasing complexity of the organism. This concept has been strengthened by experiments in which there was observed a restoration of carbohydrate metabolism to surviving diabetic tissues *in vitro* in a simple Ringer-phosphate system without the aid of insulin.

These experiments are presented under the following headings:

A. The Theories of the Pasteur Reaction
B. Lactic Acid as an Obligatory Intermediary in Oxidation of Carbohydrate
C. Source of Energy for Lactic Acid Resynthesis
D. Carbohydrate Oxidation and the Lactic Acid Enzyme System as Influenced by the Removal of the Pancreas and the Pituitary
E. The *In Vitro* Action of Insulin on Minced Muscle

F. The Restoration of Carbohydrate Oxidation to Diabetic Tissue *In Vitro* in the Absence of Insulin

A. The Theories of the Pasteur Reaction

In 1876, Pasteur, working with yeast, announced his fundamental discovery of the relation between oxidation and fermentation as alternate sources of energy for cellular metabolism under different environmental conditions. For many years thereafter little progress was made on this important problem, except for the recognition that lactic acid was concerned in the metabolism of a number of animal tissues. In 1907, Fletcher and Hopkins drew attention to the importance of the lactic acid cycle for muscle. Further impetus was given to the study of this phase of intermediary metabolism by the work of Warburg on tumors and of Hill and Meyerhof on muscular contraction. Unfortunately, however, while many of its problems still remained unsolved, attention was diverted from the lactic acid cycle by Lundsgaard's discovery that its function was subsidiary to the creatin-phosphate mechanism.

During this period the original interpretation of the Pasteur effect has been modified on the basis of newer knowledge of lactic acid metabolism. The three major theories as to the nature of the Pasteur reaction are given in Fig. 1 [modified from Burk (8)].

UNITARY THEORY:

$$\text{Carbohydrate} \rightarrow \text{Intermediary} \overset{O_2}{\rightarrow} CO_2 + H_2O$$

MEYERHOF CYCLE THEORY:
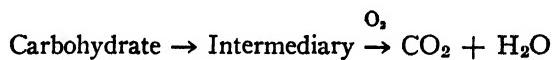
$$\text{Carbohydrate} \rightarrow \text{Intermediary}$$
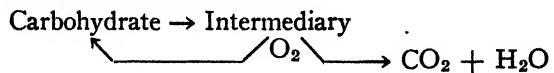
LIPMANN THEORY:

Oxidative inhibition of fermentation systems

$$\text{Carbohydrate} \overset{O_2}{\longrightarrow} CO_2 + H_2O$$
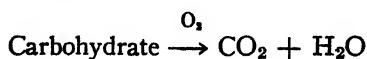
Fig. 1. Explanations of the Pasteur effect.

They need be discussed only briefly here, in as much as they have been dealt with fully by Burk (8, 8a).

The first modification of the reaction followed on the work of Pflüger and Pfeffer and is generally known as the Unitary theory. According to this concept, fermentation intermediaries arise in oxygen as well as in nitrogen and undergo subsequent oxidation to carbon dioxide and water. The later elaboration proposed by Meyerhof,

incorporating the new concept of lactic acid re-synthesis, has remained for many years the one most generally accepted. It was a real step forward to show, as he did, that the lactic acid cycle not only participated in the catabolic processes of the cell but in the anabolic processes as well. The demonstration that lactic acid appeared not only as a result of anaerobic cleavage but also under oxidative conditions, undergoing oxidation and resynthesis, was an elaboration of the original theory of great consequence for biology. Further proof of the importance of the aerobic metabolism of lactic acid was furnished by the demonstration that inhibition of glycolysis by iodoacetic acid results in the abolition of glucose oxidation but does not interfere with the oxidation of lactic acid. These experiments led to the implication that carbohydrate, whether endogenous or exogenous, must be transformed to lactic acid prior to oxidation. A second aspect of the Meyerhof theory, the resynthesis of the larger portion of the lactic acid formed to a higher carbohydrate by energy derived from the fraction oxidized, was based on chemical and respiratory studies on frog muscle.

In 1933, Lipmann (9) advanced a third theory which has gained many adherents although it still awaits experimental verification with living tissue. As a result of experiments on tissue extracts with dyes of high potentials he concluded that the presence of oxygen served to inhibit the enzyme system of glycolysis and framed his concept as follows. In nitrogen, carbohydrate is broken down to lactic acid; in the presence of oxygen, a brake is applied to the cleavage process.

The experiments reported here relate to the Meyerhof theory, and were designed to test with mammalian tissues the experimental bases on which his concept rests.

B. Lactic Acid as an Obligatory Intermediary in the Oxidation of Carbohydrate

First, I shall consider that aspect of the Pasteur effect which deals with lactic acid as a possible obligatory intermediary in the oxidation of carbohydrate. The development of this elaboration of the Pasteur effect was a consequence of studies stimulated by Lundsgaard's discovery that iodoacetic acid (IAA) inhibited lactic acid formation. Krebs (10) working with sarcoma, testis and brain, found that IAA in concentrations which inhibited glycolysis prevented the oxidation of glucose without interfering with the ability of lactate or pyruvate to maintain respiration. Confirmatory experiments on brain, relying on oxygen consumption alone, were reported by Quastel and Wheatley (11). Later, Meyerhof and Boyland (12) found that exposure of frog muscle to IAA lowered the R.Q. from the usual value of 1.0 to

the level of 0.7 to 0.8. The addition of lactate raised the quotient to above 0.9, indicating that the defect in carbohydrate oxidation produced by IAA did not reside in the mechanism for oxidizing lactic acid. All these experiments led to the assumption that carbohydrate oxidation ceased whenever the formation of lactic acid was prevented. Lactic acid was apparently a necessary intermediary in the pathway of sugar oxidation, occurring not only under anaerobic but under oxidative conditions as well.

There were, however, other experiments which suggested that this might not inevitably be the case. In 1925, Loebel (13) observed that brain cortex oxidized fructose without producing lactic acid from it under anaerobic conditions. Lundsgaard (14) was also aware of the fact that properly chosen concentrations of IAA, sufficient to suppress glycolysis, interfered very little with the rate of oxygen consumption. Unfortunately, respiratory and chemical data to indicate the nature of the foodstuffs oxidized under these conditions were not obtained. Later, Stannard (15) and Saslow (16) reported the maintenance of carbohydrate oxidation in normal and caffeinized frog muscle poisoned with IAA and iodoacetamide. Respiratory quotients between 0.90 and 1.00 were observed even when anaerobic glycolysis was completely abolished. Another dissociation of glycolysis and oxidation was obtained by Himwich and Fazekas (17), and by Baker, Fazekas and Himwich (18), who were able to stop the oxidation of lactic acid in brain tissue with nicotine without entirely suppressing glucose oxidation.

Our own experiments (19, 20) on the effect of IAA on carbohydrate metabolism have been carried out on mammalian tissues which included cardiac, smooth and skeletal muscle, and brain cortex from cats and dogs. Certain precautions were taken to insure experimental conditions free from several possible sources of error. Prior to the experiment proper, the tissues were aerated in a Ringer solution to permit the loss by diffusion of preformed lactic acid which might have accumulated in the tissues during their preparation. A second precaution was aimed at avoiding nonspecific toxic effects of IAA. This consisted of the use of concentrations just sufficient to produce a virtual inhibition of glycolysis. This concentration differed with each tissue: for skeletal muscle strips it was 1:10,000; for smooth muscle, 1:10,000 to 1:20,000; for cardiac muscle and brain, 1:75,000 to 1:100,000. Finally, in addition to measurements of oxygen consumption, respiratory quotients were determined and chemical balances carried out. The results are summarized in Table I.

Each of these mammalian tissues retained its

TABLE I

Effects of iodoacetic acid on metabolism of mammalian tissues.

Tissue	R.Q.				Q_{O_2}* (mm.³/mg./hr.)				Inhibition of glycolysis	Concentration of iodoacetic acid
	Non-nutrient	Non-nutrient-iodoacetic acid	Glucose	Glucose-iodoacetic acid	Non-nutrient	Non-nutrient-iodoacetic acid	Glucose	Glucose-iodoacetic acid		
									p.c.	
Skeletal muscle, dog	0.86	0.89			0.31	0.21			94†	1:10,000
	0.89	0.93			0.20	0.14			98†	1:10,000
	0.94		1.03	0.97	0.26		0.26	0.17	98†	1:10,000
Smooth muscle, cat			0.97	0.95			0.25	0.27	90	1:20,000
	0.85		0.95	0.95	0.30		0.34	0.26	90	1:10,000
	0.95		1.05	1.01	0.25		0.24	0.23	94	1:10,000
Heart muscle, cat	0.72		0.81	0.85	0.68		0.67	0.71	90	1:100,000
	0.87		0.96	0.99	0.33		0.37	0.37	90†	1:100,000
Heart muscle, dog	0.95		0.91	1.01	0.92		0.94	0.46	94	1:75,000
	0.83	0.81	0.91	0.90	0.60	0.58	0.61	0.58	99†	1:75,000
Brain cortex, cat	0.96	0.95			0.45	0.42			94†	1:75,000
	0.97	0.98	0.97	1.12	0.42	0.35	0.66	0.44	97†	1:100,000
	1.06	1.09	1.02	0.96	0.34	0.30	0.61	0.47	94†	1:100,000

* All quantities are calculated per unit wet weight of tissue.
† Lactic acid was measured chemically; others manometrically.

capacity to oxidize carbohydrates, both preformed or as added glucose, in concentrations of IAA sufficient to inhibit anaerobic glycolysis 90 p.c. to 100 p.c. Generally, the influence of IAA was apparent in a moderate diminution in oxygen consumption, although this was not invariable. In cardiac and smooth muscle, added glucose actually elevated the R.Q. in the presence of IAA.

Experimental data such as these are open to criticism in so far as they do not rule out the possibility that preformed lactate, increased oxidation of protein, or delayed aerobic penetration might have been responsible for the R.Q.'s obtained. These possible sources of error were investigated by chemical analyses of the systems under study.

The factor of the preformed lactate as a possible substrate was dealt with by preliminary aeration which reduced the lactic acid content to values which could account for only a small fraction of the carbohydrate oxidation in the tissues. Chemical analyses of the ammonia formed in the course of the experiment ruled out an increased protein metabolism as the source of energy and high respiratory quotients. The glucose balance sheet had even more direct bearing. The glucose which disappeared from the system accounted almost quantitatively for the level of the respiratory quotient as well as for that portion of the oxygen consumption calculated to be derived from carbohydrate oxidation.

The question of penetration could not be answered as definitely, although there was good reason to infer that aerobic penetration of IAA was as good as anaerobic. Crabtree and Cramer (21) had found that IAA caused as prompt and

complete an inhibition of aerobic as of anaerobic glycolysis in tumor tissue. To deal with the factor of penetration, we subjected tissues to a preliminary exposure to IAA under anaerobic and aerobic conditions until anaerobic glycolysis had been abolished. These experiments, one of which is illustrated in Fig. 2, agreed with the previous ones in demonstrating the ability of mammalian tissues to oxidize carbohydrate while under the influence of IAA.

This separation of glycolysis and oxidation by the use of a poison would gain added significance if it could be brought about by more normal means. A procedure of this kind was possible in the case of minced mammalian brain cortex. If this tissue is aerated in Ringer solution for a half hour, freely diffusible substrates such as glucose and lactic acid are washed out. The rate of respiration is diminished, but the R.Q. remains close to unity. In the presence of oxygen, the addition of small amounts of glucose (as little as 10 to 25 mg. p.c.), causes a sharp rise in oxygen consumption which approaches a maximum with 25 mg. p.c. Addition of these same concentrations of glucose under anaerobic conditions causes much less of an increase in glycolysis. As a result there exists a wide range over which the oxidation of carbohydrate cannot be accounted for, except in part, by the formation of lactic acid as an intermediary. A typical experiment is shown in Fig. 3.

One may infer from these experiments that a common pathway for oxidation and glycolysis down through the lactic acid state is not obligatory for mammalian tissues. That such a pathway may exist and diverge higher up is, of course, not ruled out. The point of divergence is prior to the formation of pyruvic acid, as shown by our failure in other experiments to find this metabolite
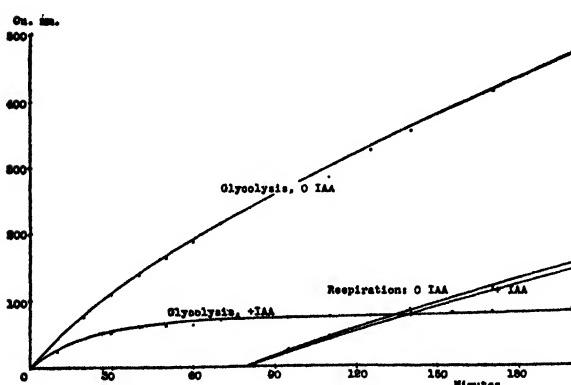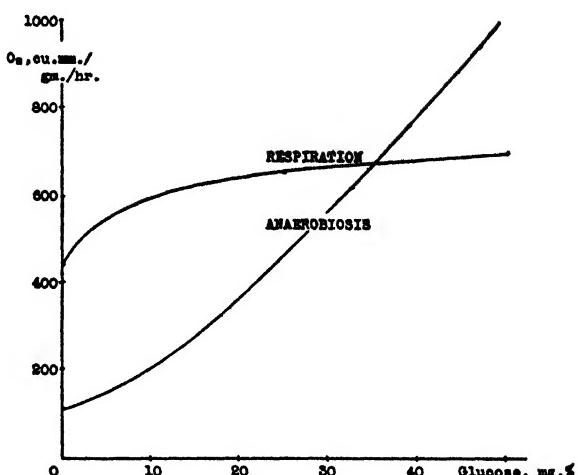
Fig. 3. Relation of aerobic and anaerobic activity of brain cortex in the presence of increasing amounts of glucose. The respiratory quotient in all the concentrations of glucose used, and in the absence of substrate, was unity. The area between the curves represents oxidation of carbohydrate for which lactic acid could not serve as an intermediary.

accumulating in the presence of IAA. The evidence from tissue extract experiments would place the point of divergence at or above triose-phosphate. The work of Cori, Cori and Hegnauer suggests that under the conditions of IAA poisoning, hexosemonophosphate might be the material oxidized (28). It is impossible to assert from these experiments that during the normal metabolism of the cell the formation of lactic acid and its subsequent oxidation and resynthesis is not the usual course of carbohydrate oxidation; but it seems reasonable to assume that alternate pathways do exist and can be brought out by the means we have employed.

The bearing of these experiments on the validity of the Lipmann *versus* the Meyerhof concept of the lactic acid cycle is not direct. By offering no support to the view that lactic acid is an obligatory intermediary they indirectly favor the Lipmann concept.

In this connection, brief reference may be made both to experiments showing an inhibitory effect of IAA on phosphorylation and to those indicating that phosphorylation is a necessary preliminary to the utilization of glucose. In the light of our experiments, these two conclusions are not compatible, since not only is preformed carbohydrate oxidized in the presence of IAA, but added glucose disappears and raises the respiratory quotient. We must, therefore, infer either that phosphorylation is not a necessary step in the oxidation of glucose, or that in the concentrations used in our experiments IAA did not interfere with phosphorylation. It may well be that the much higher

Fig. 2. Progress of respiration of cat smooth muscle (intestine) following preliminary exposure to iodoacetic acid sufficient to inhibit glycolysis. R.Q. in glucose, 0.95; in glucose plus IAA (1:25,000), 0.94. The measurement of respiration was started at 80 minutes when glycolysis was reduced to zero.

concentrations of IAA necessary to inhibit phosphorylation in tissue extracts set up chemical conditions of a different character from those under which the poison acts in the intact cell when the weaker concentrations which suffice to inhibit glycolysis are employed.

C. *The Source of Energy for Lactic Acid Resynthesis*

The second idea-implicit in the Meyerhof theory of the Pasteur reaction is the obligatory linkage of lactic acid resynthesis to lactic acid or carbohydrate oxidation. The original data on which this concept was based were derived from experiments on frog muscle, the R.Q. of which was always found to be unity, indicating exclusive oxidation of carbohydrate. The conclusions drawn from Meyerhof's experiments were that lactic acid either added to the muscle or formed by prolonged stimulation disappeared by two routes: the one, oxidation accounting for approximately one-quarter of the lactic acid formed; the other, its resynthesis into glycogen and intermediary carbohydrates, accounting for the remaining fraction. The ratio between these two processes Meyerhof called the lactic acid oxidative quotient. This quotient was around 4:1 in normal frog muscle; that is, after stimulation or in the presence of added lactate, four times the amount of lactic acid disappeared in oxygen than could be accounted for by oxidation alone.

Meyerhof derived his lactic acid oxidative quotient from either of two formulas given below. The *direct* quotient was obtained under aerobic conditions; the *indirect,* partly under aerobic and partly under anaerobic conditions.

to correct for the carbohydrate fraction of either the extra or the total oxygen consumption, depending upon whether the oxidative quotient is direct or indirect. This correction has been made in Table V and Table VIII by means of the following formula:

3. *Oxidative quotients corrected for R.Q.'s less than 1.00*

In Equations 1 and 2, multiply the denominators by

$$\frac{\text{observed R.Q.} - 0.70}{1.00 - 0.70}$$

This correction for the level of the respiratory quotient is particularly necessary under experimental conditions in which the addition of lactate increases oxygen consumption without elevating the level of the respiratory quotient.

The first group of experiments to be reported were carried out on excised muscle strips and slices of cardiac tissue from normal well-fed animals. The method developed in this laboratory (23) for obtaining muscle strips may be briefly described (Fig. 4). A long neck muscle is pinned down to a paraffin block and kept warm with Ringer solution. Individual muscle fibers are then teased out carefully, cutting the connective tissue and avoiding any trauma to the muscle proper. In this way individual muscle fibers are obtained which maintain respiration at a uniform rate for hours, have no aerobic glycolysis, and can be stimulated for long periods of time with uniform results (24).

The experimental procedure was as follows.

1. *Direct or aerobic oxidative quotient*

$$\text{Ox. Q.} = \frac{\text{HL disappearing in lactate} - \text{HL disappearing in non-nutrient medium}}{\text{HL equivalent of extra oxygen consumption in lactate}}$$

2. *Indirect or anaerobic oxidative quotient*

$$\text{Ox. Q.} = \frac{\text{HL appearing in nitrogen} + \text{HL disappearing in oxygen}}{\text{HL equivalent of total oxygen consumption}}$$

In these formulas the assumption is inherent that the tissue is burning carbohydrate exclusively; this seemed to be valid for frog muscle. However, Gemmill (22) and later Saslow (16) and Stannard (15) reported R.Q.'s of 0.9 and even lower with frog muscle. Studies in this laboratory on mammalian skeletal muscle have shown that the R.Q. of this tissue will vary from 0.72 to 1.00, depending upon the nutritional state of the animal. These wide variations in the R.Q. necessitated a change in the formula used by Meyerhof in order

Prior to the actual determination, the tissues were aerated for 30 to 60 minutes in a Ringer solution to remove the lactic acid which had accumulated during their preparation. Otherwise the accumulation is often great enough to cause a basal respiration so high that it can no longer be raised by further additions of lactate. Racemic sodium lactate in M/80 concentration was employed throughout, and lactic acid changes determined chemically on the same tissues on which the respiratory measurements were made. All the
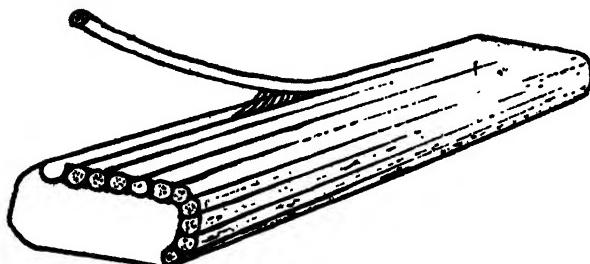
Fig. 4. Method of preparation of muscle strips for microrespiratory studies. Critical thickness,

$$r = 2 \sqrt{c_e \frac{D}{A}}$$

oxidative quotients reported were derived from chemical analyses.

Table II and Table III summarize the results obtained with skeletal muscle strips from normal well-fed dogs (25). The direct oxidative quotients averaged 4.3; the indirect, 3.3. These values are in accord with those of Meyerhof on frog muscle, and might indicate a similar mechanism in mammalian muscle for resynthesis of lactic acid. Lactic acid brings about a marked stimulation of respiration, but since the respiratory quotients in lactate tended to fall somewhat below the basal levels, there is no certainty that the increased oxygen consumption in the presence of lactate was due to the actual oxidation of lactic acid. The results of the experiments shown in Table II and Table IV would suggest that the stimulation was nonspecific.

When the nutritional state of the animal was varied by fasting, it became apparent that the values for the oxidative quotient were not as fixed as Meyerhof had assumed, but varied with different levels of the R.Q. The R.Q.'s in turn were dependent on the nutritional state of the animal. The data in Table IV have been arranged on the basis of the level of R.Q.'s, which varied from that of pure carbohydrate to pure fat oxidation. At the higher R.Q.'s the oxidative quotients were low and of the expected order of magnitude. With the lower basal R.Q.'s the values for the oxidative quotient rose, and below 0.80 values were obtained which exceeded the theoretical if we assume that all the energy for resynthesis must be derived from the oxidation of lactate or equivalent carbohydrate. Burk (26) has calculated that the maximum theoretical ratio is 10 to 1, providing the process is 100 p.c. efficient. Oxidative quotients above 10 must be interpreted as indicating that foodstuffs other than carbohydrate afforded energy for resynthesis. This evidence that fat, or the fatty acid portion of the protein molecule, could provide energy for resynthesis is

TABLE II

Oxidative quotient of washed muscle strips of the normal dog. Derived from aerobic disappearance of lactic acid. Effect of lactate on R.Q. and respiration.

Two hour periods.

Experiment	NaL conc. mg./100 cc.	Oxygen Consumption			Respiratory Quotient			Oxidative Quotient
		Non-nutrient	With lactate	Change	Non-nutrient	With lactate	Change	
		cc. per moist gm.	cc. per moist gm.	p.c.				
1	165	0.45	0.57	+27				7.2
2	90	0.42	0.55	+31				
3	145	0.48	0.65	+36	0.85	0.85	0.00	6.8
4	170	0.43	0.60	+40		0.88*		4.1
5	155	0.46	0.66	+44	1.01	0.96	—0.05	3.5
6	170	0.38	0.57	+50	0.96	0.92	—0.04	2.0
7	135	0.57	0.99	+74	1.00*			2.4
Av.	149 (app.M/80)	0.46	0.66	+44	0.94	0.91	—0.03	4.3

* Excluded from averages.

TABLE III

Oxidative quotient of washed muscle strips of the normal dog. Derived from anaerobic production of lactic acid in saline-phosphate solution.

Two hour periods.

Experiment	Control Tissue in Oxygen			Change in Tissue Lactic Acid		Oxidative Quotient
	R.Q.	Oxygen consumption	HL equivalent of total oxygen consumption	In oxygen	In nitrogen	
		mm.³ per moist gm.	mg. per moist gm.	mg./gm.	mg./gm.	
5	1.01	380	0.51	−0.41	+0.89	2.6
8*	0.95	410	0.55	+0.03	+1.74	3.1
9*	0.94	780	1.04	−0.40	+3.40	3.7
10†	0.98	290	0.39	−0.18	+1.33	3.9
Av.	0.97					3.3

* Received 0.2 per cent glucose. Experiments 5 and 10 done in saline-phosphate without the addition of glucose.
† 1 hour run.

TABLE IV

The respiratory metabolism and lactic acid oxidative quotient of muscle strips of the normal (fed and fasted) dog. (Derived from the aerobic disappearance of lactate.)

Experimental Condition	Oxygen Consumption/2 hr./gm.			Respiratory Quotient			Aerobic Glycolysis mg./gm./2 hr.	Meyerhof Quotient
	Non-nutrient	Lactate M/80	Change p.c.	Non-nutrient	Lactate M/80	Change		
Normal fed	0.46	0.66	+44	1.01	0.96	−0.05	−0.90	3.5
Normal fed	0.57	0.99	+74	1.00	—	—	−1.33	2.4
Normal fed	0.38	0.57	+50	0.96	0.92	−0.04	−0.50	2.0
Normal fed	0.43	0.60	+40	—	0.88	—	−0.90	4.1
Normal fed	0.48	0.65	+36	0.85	0.85	±0	−1.50	6.8
16 day fast	0.47	0.51	+ 9	0.82	0.76	−0.06	−0.70	13.0
46 day fast	0.48	0.58	+21	0.79	0.82	+0.03	−1.12	8.8
14 day fast	0.47	0.56	+19	0.77	0.79	+0.02	−2.0	14.9
54 day fast	0.44	0.47	+ 7	0.72	0.73	+0.01	−0.72	14.5

Maximum theoretical oxidative quotient = 10/1

in accord with observations on the whole animal showing resynthesis at all levels of the respiratory quotient.

The stimulating effect of lactic acid on respiration also varied with the basal respiratory quotient; as the R.Q. fell the stimulating effect was diminished. This falling off in stimulation of respiration is accompanied by a diminution in the lactic acid dehydrogenase content of the tissues of fasted animals as measured by the Thunberg methylene blue technique (unpublished experiments).

The experiments shown in Table IV bring additional support to the view that the increased oxygen consumption of skeletal muscle in the presence of lactate is not attributable to the oxidation of lactic acid. Added lactate may be considered a metabolic load which is dealt with by an increase in the rate of respiration but with no qualitative change in the character of the foodstuffs oxidized.

A second type of tissue, cardiac muscle, has also been studied with the same technique. Lactic acid oxidative quotients of the same order as those of skeletal muscle were obtained with the normal well-fed dog. There was one difference between these two types of muscle, namely, that lactate not only stimulated respiration but also brought about a corresponding elevation in the respiratory quotient. In cardiac muscle, therefore, the extra lactic acid consumption is apparently due to the oxidation of this substrate.

It must be stressed at this point that inasmuch as carbohydrate balances were not done, the use of the term "resynthesis" is an assumption. All that can be stated definitely is that lactic acid, as such, disappeared from the system in greater amounts than could be accounted for by oxidation. Although Meyerhof's original data have been criticized by Burk (8) there seems to be sufficient evidence to indicate actual formation of glycogen *in vitro* from lactic acid both in frog muscle (27, 28) and in liver (29). However, in view of the variations in behavior from tissue to tissue, it would be desirable to determine in both mammalian skeletal and cardiac muscle whether the disappearance of lactate is accompanied by the formation of carbohydrate. Such experiments are now in progress.

The experiments reported above (Table V) have a bearing on the second aspect of the Meyerhof concept of the Pasteur reaction. The results are contrary to the theory that resynthesis of lactic acid is necessarily linked with the oxidation of lactic acid or equivalent carbohydrate. They suggest that non-carbohydrate substances are equally able to afford energy for this process.

D. Carbohydrate Oxidation and the Lactic Acid Enzyme System as Influenced by the Removal of the Pancreas and the Pituitary

With the data available on the carbohydrate-oxidizing capacity and lactic acid oxidative quotients of normal cardiac and skeletal muscle, com-

TABLE V

Respiratory metabolism and lactic acid oxidative quotient
of excised cardiac tissue of the normal dog.

Dog	Oxygen Consumption cc./gm./hr.			Respiratory Quotient			Lactic Acid	Oxidative Quotient	
	Non-nutrient	Lactate M/80	Change (p.c.)	Non-nutrient	Lactate M/80	Change (p.c.)	Disappearance mg./gm./hr.	Meyerhof	Based on R.Q.*
1	0.80	1.06	+33	0.84	0.88	+0.04	1.22	3.5	5.9
2	0.61	1.05	+72	0.89	0.90	+0.01	1.05	1.84	3.2
3	0.59	0.68	+15	0.86	0.97	+0.11	0.50	4.0	4.4
4	0.66	1.01	+53	0.83	1.01	+0.18	1.55	3.3	3.3
5	0.54	0.82	+52	0.82	0.97	+0.15	1.30	3.4	3.8
6	0.53	1.17	+121	0.80	0.95	+0.15	0.75	0.8	1.0
7	0.54	0.77	+42	0.85	0.98	+0.13	—	—	—
8	0.49	0.65	+32	0.85	0.90	+0.05	—	—	—
9	0.43	0.78	+81	0.81	0.98	+0.17	—	—	—
Av.	0.58	0.91	+56	0.84	0.95	+0.11	1.06	2.8	3.6

parative studies were carried out on tissues from dogs made diabetic by the removal of the pancreas and from Houssay animals. Prior to the removal of tissue, several of the Houssay dogs were studied in the respiratory calorimeter by Chambers, Sweet, and Chandler (30), so that a comparison could be made of the carbohydrate metabolism of the whole animal and that of the excised tissues.

The first set of experiments were carried out on skeletal muscle strips of depancreatized dogs (31). The data which are assembled in Table VI show that striking changes follow pancreatectomy.

The level of the respiratory quotient, both under basal conditions and in the presence of lactate, indicates an essentially fat type of oxidation. Stimulation of respiration by lactate was small although its effects could be seen in a slight rise in the respiratory quotient. The oxidative quotient, in contrast with that of the normal, was unity, indicating the disappearance from the system of amounts of lactic acid which could be accounted for entirely by oxidation. The capacity to resynthesize added lactate was therefore lost. The absence of any significant aerobic glycolysis

indicated that the diabetic tissue was able to preserve the small store of carbohydrate initially present, but was unable to deal with the extra lactate by resynthesis as did the normal muscle. This failure of the mechanism for resynthesis affords a partial explanation for the diminishing glycogen supply of the diabetic skeletal muscle.

Further evidence of damage to the lactic acid enzyme system in diabetic skeletal muscle is furnished by the low rate of anaerobic glycolysis as compared to the normal. The low basal lactic acid production in nitrogen might be explained by a low glycogen content. The failure to increase the rate of glycolysis in the presence of an abundance of glucose points to a defect of quantitative nature in the lactic acid system of this tissue.

From these experiments (Table VII) one might infer that insulin lack has a profound effect not only on carbohydrate oxidation but on the lactic acid enzyme system as well. However, experiments with diabetic cardiac muscle (32) serve to localize the effect of insulin lack more specifically to the oxidative side. Results obtained with this tissue have been assembled in Table VIII.

TABLE VI

The effect of racemic sodium lactate on the respiratory and lactic acid metabolism of excised skeletal muscle of the diabetic dog.

Animal	Period	Oxygen Consumption		Respiratory Quotient		Aerobic Glycolysis	
		Non-nutrient	Lactate M/80	Non-nutrient	Lactate M/80	Non-nutrient	Lactate M/80
	min.	cc./gm.	cc./gm.			mg./gm.	mg./gm.
206	120	0.57	0.54 − 5 p.c.	0.75	0.81	+0.17	−0.30
206	150	0.51	0.63 +24 p.c.	0.71	0.76	+0.10	0.00
207	180	0.71	0.84 +18 p.c.	0.73	0.75	+0.80	+0.50
207	150	0.47	0.54 +15 p.c.	0.69	0.81	−0.20	+0.16
212	105	0.63	0.68 + 8 p.c.	0.75	0.76	+0.14	+0.20
Total	705	2.89	3.23 +12 p.c.	0.73	0.78	+1.01	+0.56

$$\text{Oxidative quotient} = \frac{0.45}{0.46} = 1.0 \text{ derived from the following data:}$$

lactate disappearing = 0.45
lactate oxidized (3.23 − 2.89) ÷ 0.747 = 0.46

TABLE VII

The anaerobic glycolysis of excised skeletal muscle of the normal and diabetic dog.
(Expressed as mg. lactic acid formed per gm. muscle in 2 hr.)

| | | Diabetic | | | Normal | |
| | | Anaerobic Glycolysis | | | Anaerobic Glycolysis | |
Animal	R.Q.	non-nutrient	0.2 p.c. glucose		non-nutrient	0.2 p.c. glucose
206	0.75	0.75	0.96		4.26	4.71
206	0.71	0.08	0.32		3.85	3.94
207	0.73	1.20	1.20		1.45	2.05
207	0.69	0.96	0.40		1.62	2.77
212	0.75	(0.34)	... ..			
Average		0.75	0.72		2.19	3.37

The stimulation of respiration of lactic acid was somewhat less than that observed with normal cardiac muscle. The basal respiratory quotients were sufficiently high to indicate some oxidation of preformed carbohydrate; several were in the usual diabetic range. The failure of this substrate to raise the respiratory quotient was in sharp contrast to the response of normal tissue to lactate. The lactic acid oxidative quotient, particularly when calculated on the basis of the respiratory quotient, was much higher than normal and indicated retention of the capacity to resynthesize added lactic acid. This unusually high oxidative quotient may explain the fact that the glycogen stores of the diabetic heart may rise to higher than normal values. The failure of lactic acid to elevate the respiratory quotient, despite a considerable stimulation of respiration, supports the view previously expressed that this stimulation may be of a non-specific character.

TABLE VIII

Respiratory metabolism and lactic acid oxidative quotient
of excised cardiac tissue of the diabetic dog.

| Diabetic Dog | Oxygen Consumption cc./gm./hr. | | | Respiratory Quotient | | Lactic Acid | Oxidative Quotient | |
	Non-nutrient	Lactate M/80	Change (p.c.)	Non-nutrient	Lactate M/80	disappearance mg./gm./hr.	Meyerhof	based on R.Q.
1	0.79	1.03	+30	0.86	0.84	0.75	2.3	5.1
2	0.92	1.08	+17	0.79	0.73	1.10	5.2	(67)*
3	1.05	1.24	+18	0.88	0.88	1.54	6.2	10.5
4	0.79	1.18	+49	0.80	0.85	1.55	3.1	6.3
5	1.24	1.70	+37	0.82	0.84	1.42	2.3	5.1
6	0.83	1.08	+30	0.75	0.77	1.05	3.4	15.7
7	0.66	0.90	+39	0.72	0.75	.. ...	..	.. ..
8	0.62	0.95	+53	0.71	0.76	....	.....	......
Average	0.86	1.15	+34	0.79	0.80	1.24	3.8	8.6
Normal	0.58	0.91	+56	0.84	0.95	1.06	2.8	3.6

* Excluded from averages.

TABLE IX

Respiratory metabolism of cardiac tissue from diabetic dogs
in the presence of glucose.

Diabetic Dog	Oxygen Consumption cc./gm./hr.			Respiratory Quotient	
	Non-nutrient	Glucose 0.2 p.c.	Change	Non-nutrient	Glucose 0.2 p.c.
1	0.66	0.82	+26 p.c.	0.72	0.76
2	0.62	0.65	+ 5 p.c.	0.71	0.71
3	0.84	0.74	−12 p.c.	0.72	0.72
4	0.64	0.65	± 0	0.78	0.78
5	0.44	0.47	+ 6 p.c.	0.76	0.71
6	0.54	0.60	+11 p.c.	0.84	0.73
7	0.71	0.82	+16 p.c.	0.74	0.73
Average	0.64	0.68	+ 6 p c	0.75	0.73
Normal	0.49	0.53	+ 8 p.c.	0.84	0.97

In these experiments, in contrast with skeletal muscle, diabetic cardiac muscle retains the ability to glycolyze in normal fashion and to increase its formation of lactic acid from added glucose. The failure to oxidize added lactic acid is also accompanied by an inability to oxidize added glucose.

The comparison of the metabolism of cardiac and skeletal muscle shows the danger of generalizing from studies on one type of tissue. From the results with skeletal muscle one might infer that insulin lack specifically damaged both the oxidative and lactic acid enzyme systems. The results with cardiac muscle make it clear that glycolysis and lactic acid resynthesis may go on normally even in the absence of insulin, whereas the oxidation of carbohydrates suffered from this lack. Experiments with brain cortex show apparently no interference with either system in the absence of insulin. For the present this tissue

TABLE X

The respiratory metabolism and lactic acid oxidative quotients of the Houssay
dog obtained with washed strips of excised skeletal muscle.

Exp.	No. days since		Respiratory Quotient			Oxygen Consumption-2 hrs.			Lactic Acid Oxidative Quotient
	Hypophy-sectomy	Pancrea-tectomy	Non-nutrient	M/80 lactate	Change	Non-nutrient	M/80 lactate	Change	
						cc./moist gm.		p.c.	
1	9+	5	0.80	0.82	+0.02	0.50	0.55	+11	0.3
2	19+	10	0.74	0.79	+0.05	0.41	0.55	+35	1.57
3	27	9	0.81	0.87	+0.06	0.39	0.51	+31	5.94
4	35	17	0.81	0.93	+0.12	0.32	0.43	+37	4.21
5	27	13	0.79	0.90	+0.11	0.33	0.39	+18	13.1
6	34	20	0.82	0.84	+0.02	0.42	0.59	+35	2.4
7	35	12	0.83	0.86	+0.03	0.35	0.53	+49	1.8
Average			0.80	0.86	+0.06	0.39	0.51	+31	4.1

must be regarded as a special case in which carbohydrate metabolism is entirely independent of insulin. The significance of the results with cardiac muscle lies in the fact that a definite defect on the oxidative side does exist, hence it is a tissue dependent on insulin for its normal metabolism.

Comparable studies on tissues excised from Houssay dogs afforded the opportunity to examine the oxidative and glycolytic mechanisms freed from the influence of insulin and the pituitary hormones. The first group of experiments (Table X) were carried out with excised skeletal muscle (33).

The results, as far as the respiratory quotients and lactic acid stimulation are concerned, were intermediate between the normal and the diabetic and corresponded with the level of carbohydrate metabolism of the intact animal. The lactic acid oxidative quotients varied considerably, but indicated the retention of the mechanism for resynthesis in contrast with purely diabetic tissue. In the absence of insulin and the pituitary hormones, there is a return to normal of the mechanism for lactic acid resynthesis and a partial restoration of the capacity to oxidize carbohydrates.

With excised cardiac muscle from the Houssay dog (34) the return to normal was even more striking, particularly on the oxidative side. Respiratory quotients of an entirely normal character, both under basal conditions and in the presence of glucose and lactate, were obtained. Lactic acid oxidative quotients returned to the normal levels. In the absence of insulin, as far as can be judged from the data on hand, an entirely normal type of carbohydrate metabolism takes place in excised cardiac tissue from the Houssay animal. The extent of the return to normal is far greater than we should have anticipated from the whole animal, as is evident from the data of Chambers, Sweet, and Chandler (30) as presented in Table XI. The significance of this phenomenon is dealt with in Section F.

The shifts in the lactic acid resynthesis mechanism observed in these different hormonal states have a counterpart in the carbohydrate content of the tissues of the whole animal. The total carbohydrate content of cardiac tissue from normal, diabetic, and Houssay dogs is presented in Table XII. The higher carbohydrate levels in the diabetic heart may be a result of the higher oxidative quotients observed with this tissue *in vitro*.

The metabolism of pyruvic acid is also influenced by the hormonal state of the animal. A few of the results obtained with Barker and Malam (unpublished data) are presented in Table XIII. Removal of the pancreas diminishes stimulation of respiration by this substrate, and there is partial restoration in the tissues of the Houssay dog. The respiratory quotient with pyruvic acid is 1.34 for normal tissues. This would indicate that something in addition to oxidation is taking place, since the respiratory quotient for pyruvic acid is 1.20. In diabetic tissue the respiratory quotient is the theoretical one; in tissue of the Houssay dog the quotient is again elevated to the levels found with the normal dog. The data pre-

TABLE XI

The respiratory metabolism and lactic acid oxidative quotient of excised cardiac tissue of the Houssay dog.

Animal	Oxygen Consumption cc./gm./hr.			Respiratory Quotient			Lactic Acid Oxidative Quotient	R.Q. of Whole Animal in Calorimeter*	
	Non-nutrient	Lactate M/80	Glucose 0.2 p.c.	Non-nutrient	Lactate M/80	Glucose 0.2 p.c.		Basal	After Glucose
202	0.40	0.80 +100 p.c.		0.83	1.00	—	2.5	0.78 0.79	0.81, 0.83 0.83
208	0.41	0.62 +51 p.c.	0.42 +2 p.c.	0.86	0.95	0.92	3.6	0.78	0.77
209	0.29	0.54 +86 p.c.	0.32 +9 p.c.	0.98	0.95	0.98	2.8	0.78 0.79	0.84 0.86
217	0.26	0.45 +73 p.c.	0.28 +8 p.c.	0.85	0.91	0.95	2.1	0.76	0.76, 0.79
Av.	0.34	0.60	0.34	0.88	0.95	0.95	2.8		

* Chambers, Sweet and Chandler.

TABLE XII

Total fermentable carbohydrate and lactic acid in cardiac muscle of the dog.

Normal mg./gm.	Diabetic mg./gm.	Houssay mg./gm.
5.3	12.8	5.4
6.5	12.7	15.1
6.5	9.3	6.6
6.2	14.8	
7.2	11.0	
7.0	15.1	
10.6	14.9	
12.6	8.1	
9.5	14.5	
	18.2	
	13.1	
	15.7	
	12.1	
	17.3	
Av. 7.9	13.5	9.0

sented are incomplete in that we are uncertain as to the significance of the respiratory quotients being above the theoretical in the tissues from the normal and Houssay dogs. The respiratory quotients are presented to indicate that in diabetes a change occurs in pyruvic acid metabolism which returns to normal when the pituitary is removed.

These results throw some light on the mode of action of hormones on carbohydrate metabolism. Insulin and the pituitary and adrenal cortical hormones may be conceived of either as essential components of the enzyme systems controlling carbohydrate metabolism, or as non-essential regulating influences which act by accelerating or inhibiting enzymatic processes. The latter possibility is favored by the return to normal of carbohydrate metabolism in the tissues of the Houssay dog. The actual enzyme systems appear to be essentially independent of the hormones. The results of these experiments may also prove of value in arriving at a common denominator underlying the essential defect resulting from insulin lack. They suggest that the primary effect of this hormone is not upon the lactic acid enzyme system but rather on the oxidative mechanism, since the former may continue unimpaired in a tissue which has lost its capacity to oxidize carbohydrate.

TABLE XIII

The effect of pyruvate on the respiratory metabolism of excised cardiac muscle.

	Oxygen Consumption			Respiratory Quotient	
	Non-nutrient	Pyruvate 0.2 p.c.	Change p.c.	Non-nutrient	Pyruvate
Normal Dog	0.54	1.12	+107	0.85	1.37
	0.49	1.61	+228	0.85	1.31
	0.43	1.01	+135	0.81	1.35
			+157 p.c.		1.34 av.
Diabetic Dog	0.66	1.05	+ 61	0.72	1.18
	0.62	0.81	+ 32	0.71	1.19
	0.84	1.15	+ 37	0.72	1.20
	0.44	0.57	+ 28	—	—
			+ 39 p.c.		1.19 av.
Houssay Dog	0.40	0.76	+ 90	0.83	1.40
	0.41	0.79	+ 92	0.86	1.32
	0.29	0.61	+110	0.99	1.32
			+ 98 p.c.		1.35 av.

E. The In Vitro Action of Insulin on Minced Tissue

The data presented above would indicate that the defect in carbohydrate metabolism resulting from insulin lack primarily involves the oxidative rather than the glycolytic mechanism. In view of this, the results obtained by Krebs and Eggleston (6) with insulin on minced pigeon breast muscle are of special interest. These workers demonstrated that addition of insulin to minced pigeon breast muscle in the presence of phosphate, citrate and boiled muscle juice, brings about an increase in respiration over the controls. These experiments constituted the first *in vitro* demonstration of an insulin effect on oxidative metabolism. Since the most pronounced effect on respiration occurred in the presence of citrate, Krebs and Eggleston inferred that insulin influenced the metabolism of carbohydrate by acting catalytically on the citric acid cycle. Crystalline zinc insulin failed to produce any stimulation of respiration and was therefore considered either an unnatural or an inactive depot form of the hormone.

Because of the potential importance of these observations for elucidating the relation of insulin to sugar metabolism it was thought worthwhile to examine a variety of experimental animals (35) to determine how general this phenomenon was. The results obtained by Krebs and Eggleston on the pigeon breast were confirmed, though there were some minor differences. The increased respiration in the presence of insulin was somewhat less than they reported and crystalline insulin was found as effective as amorphous. We were also not inclined to interpret the effect of insulin on respiration as a true stimulation. As can be seen from Fig. 5 respiration falls off rapidly

in all solutions whether or not insulin is added. The insulin effect becomes manifest after about 80 to 100 minutes when the oxygen consumption has fallen to approximately one-half the initial value. From then on the effect of insulin is to delay the fall of respiration in a system in which the rate of oxygen consumption is diminishing rapidly. It would seem more accurate, therefore, to regard the increased oxygen consumption in the presence of insulin neither as a stimulation nor as maintenance of respiration, but rather as due to a lag in the rate at which it is falling off.

Repetition of these experiments with Barker, using minced skeletal muscle of the chicken, rabbit, cat and dog, and minced cardiac muscle of the dog, uniformly failed to show any increased respiration with insulin (Table XIV). Experimental conditions such as fasting and pancreatic diabetes were set up in which insulin is known to be a limiting factor. Various modifications were made in technique, such as changes in insulin concentration, in pH, in temperature, and the addition of cytochrome-c; all were ineffective. Of the variety of animals studied only the pigeon showed this insulin effect.

The localization of the reaction to pigeon breast mince led us to study some of the respiratory characteristics of this preparation. In contrast with the wide range of respiratory quotients of mammalian skeletal muscle under various nutritional conditions, that of the pigeon mince was always approximately unity under all experimental conditions, including fasts of as long as four days. The respiratory quotient of the pigeon breast *slice*, however, varied with the nutritional state. Simultaneous studies of the slice and the mince showed that mincing *per se* brings about an immediate elevation of the respiratory quotient to levels representing almost exclusive carbohydrate oxidation. This phenomenon was not confined to the pigeon mince, but was also obtained with skeletal muscle from fasted and depancreatized dogs (Table XV).

The effect of insulin on respiration of the pigeon mince is therefore only of a quantitative nature, since the mince in the absence of insulin is already oxidizing carbohydrate exclusively. Better evidence for the specific character of the insulin reaction would have been furnished by a change from a non-carbohydrate to a carbohydrate type of metabolism, as is seen in the whole animal.

The observations reported on the effects of pancreatectomy on the pigeon by Stare and Baumann (36) at this Symposium also have a bearing on the question of the specificity of the insulin effect. They found that the pigeon, like the duck (37), tolerates pancreatectomy extremely well with no significant changes in the already
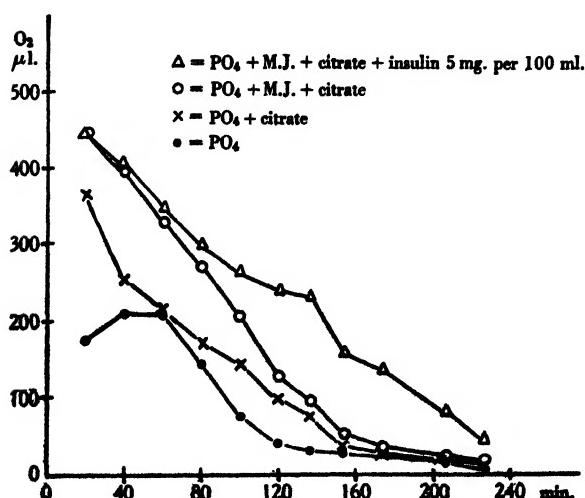
Fig. 5. Increments of respiration of minced pigeon breast muscle.

TABLE XIV

Effect of insulin on respiration of minced mammalian and chicken muscle

Tissue	Duration of exp.	Q_{O_2}		Amount and kind of insulin
		No insulin	Insulin	
	hr.	mm.³/mg./hr.	mm.³/mg./hr.	mg./100 cc.
Dog Skeletal:				
Post-absorptive	3.3	0.71	0.69	1.5, B.D.H.
			0.67	6, B.D.H.
			0.56	30, B.D.H.
			0.53	60, B.D.H.
Depancreatized-fasted	2.3	1.01	0.95	3, B.D.H.
Dog Cardiac:				
Post-absorptive	3.4	1.10	1.08	0.4, B.D.H.
			0.94	2, B.D.H.
			0.93	8, B.D.H.
Depancreatized-fasted	2.5	1.21	1.14	0.05, B.D.H.
			1.13	0.5, B.D.H.
			1.09	5.0, B.D.H.
Cat Skeletal:				
Post-absorptive	3.9	0.95	0.80	1.5, B.D.H.
			0.91	6, B.D.H.
			0.88	30, B.D.H.
			0.83	60, B.D.H.
Rabbit Skeletal:				
Post-absorptive	2.3	0.60	0.56	1.5, Lilly
			0.59	6, Lilly
			0.59	6, Lilly
			0.58	30, Lilly
			0.59	60, Lilly
Chicken Breast	2.0	1.11	0.99	0.5, Lilly
			1.07	5.0, Lilly
			1.06	25.0, Lilly

high blood sugar level. Furthermore, we have observed that fasts of as long as four days have little effect on the blood sugar of the pigeon. It is curious that an insulin effect should be observed only in the pigeon, which is apparently little dependent on insulin, and that under experimental conditions in which it constituted a limiting factor it should be absent in the tissues of animals for which the hormone is essential.

The significance of the insulin effect on the respiration of pigeon mince as exemplifying the general mechanism of insulin action is open to question in view of the observations cited above. The potential importance of this phenomenon, however, merits its further exploration, and final judgment should be deferred until more information is available.

Inasmuch as so many concepts of intermediary metabolism now current are based on results obtained with minced tissue, the experiments showing the effect of mincing *per se* on the respiratory quotient should be of interest to workers employing this procedure. Obviously, by the disruption of the cell structure more than a *quantitative* change is brought about in the character of the metabolic processes. An explosive change to a carbohydrate type of metabolism took place in a tissue which, when intact, was burning chiefly fat.

TABLE XV

The effect of mincing on the respiratory quotient of pigeon breast muscle.*

Animal	Condition		R.Q.	Q_{O_2}
1.	Fed	mince	0.89	
2.	Fed	mince	0.95	
3.	Fasted 3 days	mince	1.01	
4.	Fasted 3 days	mince	0.98	
5.	Fasted 3 days	mince	0.96	
6.	Fasted 3.5 days	mince	0.96	
7.	Fasted 4 days	mince	0.88	
8.	Fasted 4.5 days	mince	0.93	
9.	Fasted 3 days	slices	0.79	0.81
		mince	0.96	4.03
10.	Fasted 3.5 days	slices	0.87	0.58
		mince	0.96	2.21
11.	Fasted 4.5 days	slices	0.85	0.73
		mince	0.93	4.45

* All experiments were carried out in a phosphate buffer.

It is open to question whether the character of this metabolism is identical with that which takes place in the normal living cell. It may well represent an abnormal process resulting from the liberation of highly reactive substances no longer under control of the same type of chemical organization which exists in the intact cell. Only when a reaction found in a mince has been shown to exist in a living cell, does it become free from this criticism.

Not only do qualitative metabolic changes follow mincing, but quantitative changes in respiration are brought about by different methods of mincing. The results of experiments employing two different methods may be described briefly. Comparison of the respiration of a number of tissues was made employing the Latapie mincer and the homogenizer devised by Elvehjem. Two parenchymatous organs, liver and kidney, and two types of muscle, cardiac and striated, were studied. The former muscle has small cellular units; the latter, cells which extend the length of the muscle fiber. Histological studies were made at the end of each experiment to judge the extent of cellular damage. Similar studies were made of slices or strips from the same tissues.

After homogenization, sections of liver, kidney and cardiac muscle showed complete disintegration of the cells. The Latapie mince of the same tissues showed less histological damage. This histological difference was reflected in a higher rate of respiration of the Latapie mince. With skeletal muscle the same amount of structural damage was sustained from both procedures, apparently as a result of the length of the cellular units; and the rates of respiration of the two minces were identical. The superficial cell layers of the slices showed degenerative changes, but the bulk of cells in the interior remained normal in appearance. The muscle strips retained their normal structure. Respiration of the slice or strip was maintained at a uniform rate for long periods whereas the higher oxygen consumption of the mince fell off rapidly with time. In Fig. 6 are illustrated the results of short experiments with the various tissues studied.

These results point out the desirability of adopting a uniform technique if comparisons of the data obtained in different laboratories are to have any validity. The changes of both a qualitative and quantitative nature in metabolism of tissue minces should be borne in mind when interpreting data obtained with these preparations and extending them by analogy to the living cell.

F. The Restoration of Carbohydrate Oxidation to Diabetic Tissue In Vitro in the Absence of Insulin

Studies on the Houssay and adrenalectomized-depancreatized dog have furnished convincing evidence that insulin is not essential for the utilization of carbohydrate. The chief differences between Houssay and normal animals were the slower rate and lesser degree of carbohydrate metabolism of the former. It seemed likely that there still existed in the whole animal unknown factors which continued to limit the extent of carbohydrate metabolism even after the pituitary as well as the pancreas was removed. For our experiments with tissues from the Houssay animal we were fortunate to be able to work with the dogs whose metabolism had already been studied in the respiratory calorimeter by Chambers, Sweet and Chandler.

As pointed out in Section D, excised cardiac tissue from the Houssay dog had a far more normal type of carbohydrate metabolism than the animal from which it had been removed. One explanation for this difference in behavior might be the escape of the excised tissue *in vitro* from inhibiting influences present in the whole animal. It seems certain that there is a constant degradation of hormonal factors in the body which necessitates their continual replenishment. *In vitro* the tissues are cut off from any source of supply, and with sufficient time all of the hormones carried over from the animal should be used up; the tissues would then be free of their restraining
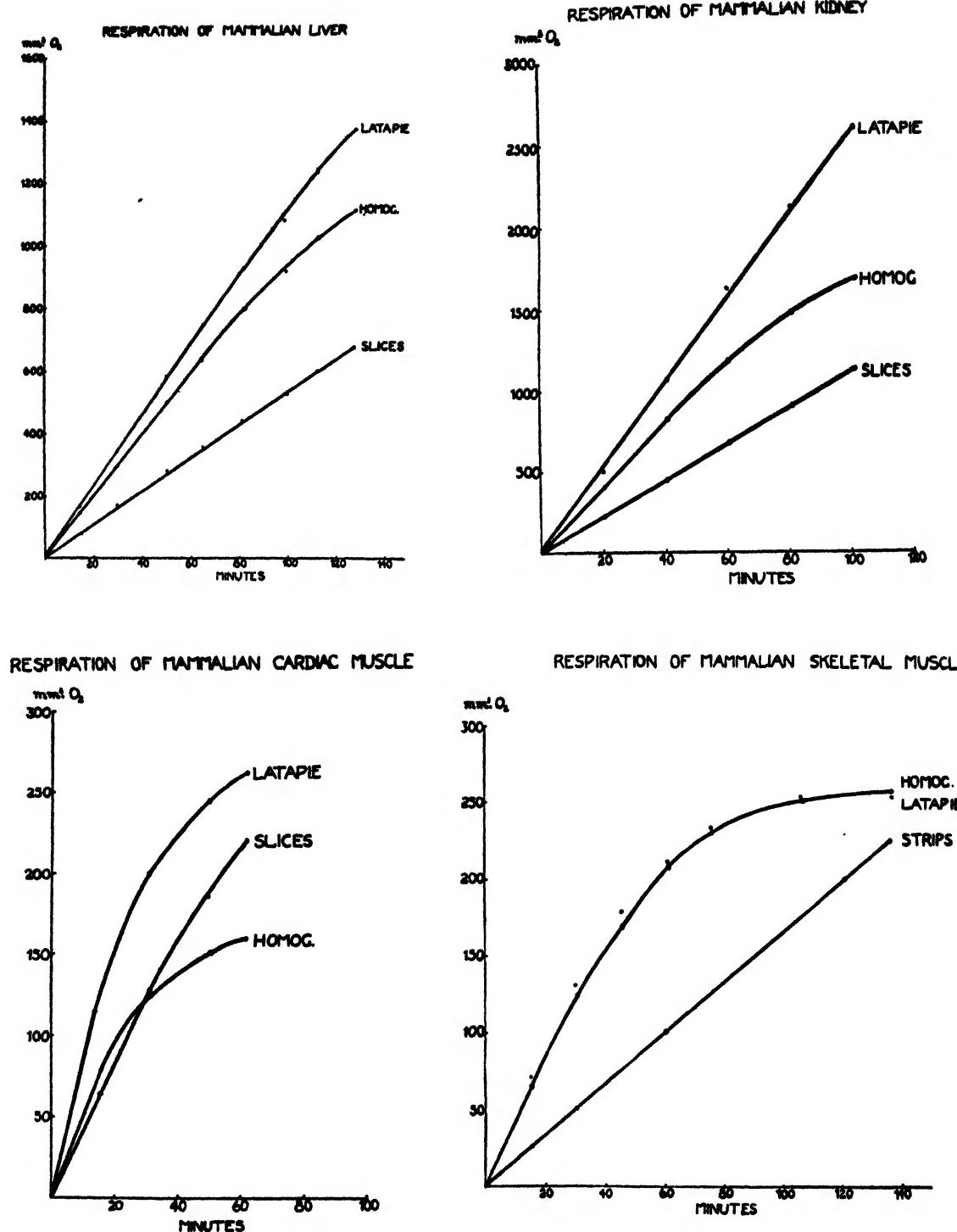
FIGURE 6.

influences. Under such conditions the primitive potentialities of the cell for the utilization of all types of foodstuffs without the assistance of hormones might manifest themselves. That these potentialities do exist, even in highly organized species, is inherent in the results of Needham (38), working with chick embryo, and the studies of Amberson and Armstrong (39) on sea urchin eggs. Both of these tissues were capable of oxidizing carbohydrates prior to the development of endocrine structures.

To test this hypothesis tissues from depancreatized dogs were removed under aseptic conditions. Respiratory studies made immediately upon removal from the body showed a diabetic type of metabolism. Other portions were incubated under sterile conditions at 37.5° C. in a Ringer-glucose-phosphate solution at pH 7.4 in an atmosphere of oxygen. Throughout the course of incubation they were shaken to maintain gaseous equilibrium, the CO_2 evolved being absorbed by alkali. Studies of the respiratory metabolism of the incubated tissue were made from time to time under the same conditions as the initial run. With time the initial diabetic level of the R.Q. rose until at the end of 10 hours the R.Q. was close to unity, indicating exclusive oxidation of carbohydrate (40). The experiments were done chiefly on slices of cardiac tissue, which invariably showed this effect, and we have also succeeded in demonstrating such a shift in excised diabetic skeletal muscle. The addition of insulin to the system did not accelerate the reversion to carbohydrate metabolism. Histological examination of the tissues at the end of incubation have shown them to retain a normal structure (Table XVI).

There are several possible interpretations of this phenomenon. It may represent an abnormal process resulting from tissue destruction or disorganization similar to the effect of mincing pigeon breast muscle, a procedure which results in an immediate elevation of the R.Q. (*cf.* Section E). The histological integrity of the tissues after incubation speaks against this interpretation. Another explanation would follow the hypothesis stated above and attribute the restoration of carbohydrate oxidation to the liberation of the tissues from restraining factors originally present. Following this concept the phenomenon would be an acute *in vitro* counterpart of the Houssay effect in the whole animal. Knowledge of the chemical processes taking place during this transition to carbohydrate metabolism would contribute greatly to our understanding of the mechanism of carbohydrate oxidation and the part played by the hormones in this process. At present we have no information as to whether the change is brought about by the formation of enzymes initially deficient or absent, or whether it results from the release of an initially adequate enzyme system from inhibitory influences.

It became of interest to determine the effects of incubation on tissue excised from normal animals (unpublished data). It is well known that normal animals do not have an exclusive carbohydrate metabolism except under special

TABLE XVI

The respiratory quotient of excised cardiac tissue of diabetic dogs following prolonged incubation in Ringer-glucose-phosphate at 37.5° C.

Dog	Medium	Respiratory Quotient			O_2 cons. cc./gm./hr.		
		Initial	After 5 hrs.	After 10 hrs.	0	5	10
1	non-nutrient	0.77	0.84	0.90	0.72	0.54	0.51
	glucose 0.2 p.c.	0.74	0.89	0.94	0.62	0.46	0.49
2	non-nutrient	0.74	0.81	0.81	0.71	0.87	0.72
	glucose 0.2 p.c.	0.73	0.82	0.88	0.82	0.89	0.68
3	non-nutrient	0.72	0.77	0.85	0.84	0.43	0.37
	glucose 0.2 p.c.	0.72	0.92	1.11	0.74	0.55	0.39
4	non-nutrient	0.78	0.82	0.78	0.64	0.95	1.00
	glucose 0.2 p.c.	0.78	0.86	1.00	0.65	1.06	0.65
5	non-nutrient	0.76	0.85	0.90	0.44	0.38	0.34
	glucose 0.2 p.c.	0.71	0.94	1.05	0.47	0.43	0.43
Average diabetic	non-nutrient	0.75	0.82	0.85	0.67	0.63	0.59
	glucose 0.2 p.c.	0.73	0.89	0.99	0.66	0.68	0.53

conditions, and that in the post-absorptive state and during brief fasts, carbohydrate oxidation is depressed even though there is an abundance of this substrate in the tissues. The present concept of inhibiting and accelerating factors controlling carbohydrate metabolism can be logically extended to the normal animal as an explanation for these facts. Experiments were therefore carried out with excised cardiac muscle from normal dogs, using the same incubation technique as with diabetic tissue, with essentially the same results (Table XVII). The initial carbohydrate metabolism was, as would be expected, greater than that exhibited by the diabetic animal. The elevation of the R.Q. occurred more rapidly but, interestingly enough, never exceeded unity. It is significant that regardless of the different initial levels, the R.Q. in both diabetic and normal tissue arrived at the same end point representing exclusive oxidation of carbohydrate. If the elevation of the quotient were the result of a non-oxidative process, as, for example, decarboxylation, we might except the quotients to rise above unity.

Extension of these studies in several other directions (41) has furnished additional support to the assumption that we may be dealing in shifts to carbohydrate metabolism of a character similar to that occurring in the living animal. The work has been greatly facilitated by the observation that when the experiments were carried out at 41° C. instead of 37.5° C., R.Q.'s of unity were reached in 4 hours as compared to 10 hours at the lower temperature. The presence of inorganic phosphate was found to be an important, and possibly a necessary, condition for the development of the reaction; when it is replaced by buffers such as glycine or sodium β-glycerophosphate, the rise in the respiratory quotient is slight or fails to occur. It seems likely, therefore, that the reaction proceeds in two steps; the first, preparatory, consisting of the removal of inhibitory influences with time; the second, the actual shift to carbohydrate oxidation under the influence of inorganic phosphate. Studies of several intermediaries involved in phosphate transfer showed no consistent changes in hexosemonophosphate or adenosine triphosphate during incubation. However, phosphocreatin invariably fell to low values as the respiratory quotient rose. This observation gains significance from the findings of Chambers, Chandler and Barker (42) that there is an increased excretion of creatin during the "pre-mortal" resumption of carbohydrate oxidation by the diabetic and fasted dog.

One hesitates to make too sweeping generalizations from experiments of this character. However, certain inferences appear to be justified in so far as they are supported by studies on the whole animal. One is that insulin is not essential for the oxidation of carbohydrate by mammalian tissue. It follows that the set of reactions which bring about the oxidation of carbohydrate may be divided into two groups, one of which consists of the essential enzymatic processes by which oxidation is brought about, and the other, of factors

TABLE XVII

The respiratory quotient of excised cardiac tissue of normal dogs following prolonged incubation in Ringer-glucose-phosphate at 37.5° C.

Dog	Medium	Respiratory Quotient					O_2 cons. cc./gm./hr.		
		Initial		After 5 hr.		After 10 hr.	0	5	10
1*	non-nutrient	0.83		—		—	0.61	—	—
	glucose 0.2 p.c.		0.94		—	1.00	0.65	—	0.69
2	non-nutrient	0.84		0.90	†	—	0.81	0.67	—
	glucose 0.2 p.c.		0.85		0.97	—	0.76	0.99	—
3	non-nutrient	0.81		0.90		0.89	0.47	0.68	0.57
	glucose 0.2 p.c.		0.89		0.92	1.00	0.53	0.74	0.86
4	non-nutrient	0.79		0.93		—	0.62	1.36	—
	glucose 0.2 p.c.		0.88		0.97	0.98	0.71	1.59	0.97
Aver.	non-nutrient	0.82		0.91		0.89	0.63	0.90	0.57
	glucose 0.2 p.c.		0.89		0.95	0.99	0.66	1.11	0.84

* Fasted 2 days
† 6 hrs. incubation

which influence their rate. The hormones would fall into the latter group, with insulin as an accelerating factor and those derived from the pituitary and adrenals as inhibitors. According to this theory, the role of the hormones controlling carbohydrate metabolism would differ from that of the vitamins now being recognized as essential components of the reactions to which they are related.

The demonstration that carbohydrate metabolism can be restored to diabetic tissues *in vitro* has a bearing on the long-standing controversy over the *underutilization* and *overproduction* theories of diabetes. The former holds that there is a generalized suppression of carbohydrate oxidation in pancreatic diabetes. The *overproduction* theory restricts the defect in diabetes to the liver, and assumes an overproduction of sugar from fat in this organ. Carbohydrate oxidation is going on normally elsewhere, and the hyperglycemia and resultant glycosuria are due to the inability of the peripheral tissues to deal with the surplus sugar formed.

All *in vitro* studies of the respiratory metabolism of diabetic tissue such as muscle and kidney have yielded respiratory quotients indicating virtual suppression of sugar oxidation, with one exception: brain oxidizes carbohydrate exclusively both *in vivo* and *in vitro*. These results would necessitate a radical alteration in the *overproduction* theory, whereby the overproduction of sugar from fat would take place in all the tissues of the body rather than in the liver alone. There is no experimental support for this assumption.

The weight of evidence supplied by the *in vitro* experiments on diabetic tissue cited above, the demonstration of the transition from a fat to a carbohydrate type of metabolism on incubation, together with the mass of careful observations on the whole animal support the concept of a generalized action of insulin on the organism and the *underutilization* theory of diabetes.

Summary

Experiments have been described relating to the oxidative and lactic acid metabolism of normal dogs and dogs in which hormonal deficiencies have been established by the removal of the pancreas or pituitary, or both. The aim of the work was to set up criteria for evaluating the effects of these hormonal influences on carbohydrate metabolism and to inquire into the nature of the relation of the hormones to the reactions essential to the utilization of this foodstuff. In the course of the experiments the bases of the Meyerhof concept of the Pasteur reaction were examined.

It was found that lactic acid was not a necessary intermediary for the oxidation of carbohydrate and that energy for lactic acid resynthesis *in vitro* could be furnished by a variety of foodstuffs. These results cast doubt on the validity of the Meyerhof interpretation of the Pasteur reaction.

A definite impairment of carbohydrate oxidation was observed in diabetic tissue *in vitro*. The lactic acid enzyme system was shown to function in a normal manner in diabetic tissue unable to oxidize carbohydrate. Therefore, the primary defect, which results from the lack of insulin in diabetes, apparently lies in the oxidative mechanism.

Removal of both the pituitary and the pancreas brings about a restoration of normal carbohydrate metabolism *in vitro*. The inference from these experiments is that carbohydrate utilization *per se* is fundamentally independent of hormonal influences. In the course of evolution the latter have developed as regulating mechanisms essential to the proper function of the living animal.

This hypothesis is in keeping with the results obtained in the whole animal from which the pancreas together with the pituitary and adrenals have been removed. It is further supported by the *in vitro* demonstration that tissues from diabetic animals regain their capacity to oxidize carbohydrates without the aid of insulin after relatively short periods of survival in a simple medium.

This concept is summarized in the following diagram :—
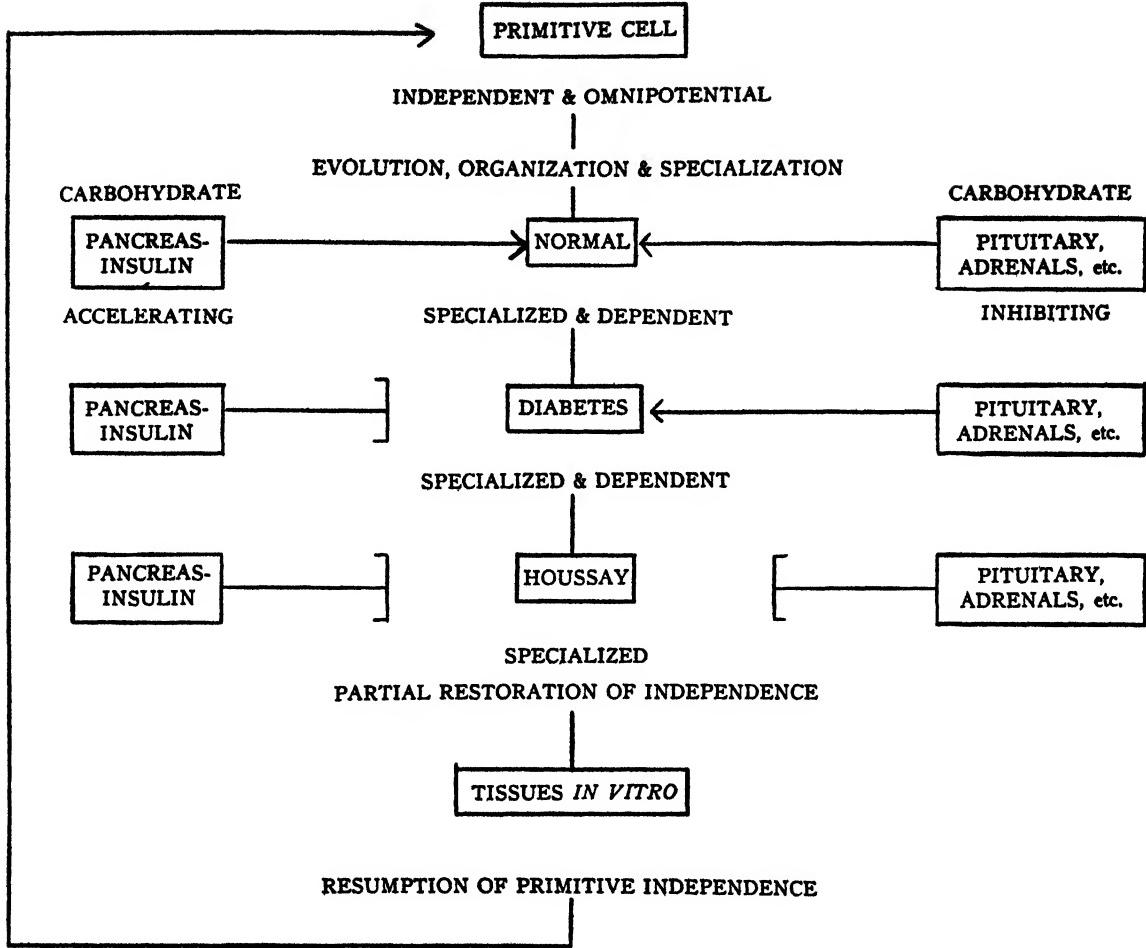
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DISCUSSION

Dr. Chambers: I am much interested in your last diagram, particularly the inhibitory factor, "and so forth", which is shown with the pituitary and adrenal. It also seems to be present in the whole animal. We have found that removal of both the pituitary and the adrenals in the depancreatized dog seems to have no greater effect on the diabetes than extirpation of either gland separately. In only two of many depancreatized dogs have we seen recovery of normal glucose oxidation, that is, respiratory quotients of approximately 1.0 after feeding glucose. Other inhibitory factors besides the pituitary and adrenal are indicated. Would you care to comment further on the "and so forth" fraction of the inhibitory substances?

Dr. Shorr: The experiments Chambers decribes suggest several possible explanations for the persistence of some limitation of carbohydrate oxidation even after the removal of both pituitary and adrenal glands from a depancreatized animal. Other ductless glands than these mentioned may participate in the inhibition of carbohydrate oxidation. A second possibility is that factors other than hormonal may be involved in the control of sugar metabolism. A third explanation, to which I lean, would place the responsibility not on any definite substances, but rather on the complexity of the living organism, whose proper functioning is dependent on a delicate integration of a variety of interrelated processes. The capacity for complete carbohydrate oxidation may exist in an animal deprived of the ductless glands as Chambers mentioned. Yet the animal may not be able to exercise this function because of non-specific factors, such as the rate of mobilization and storage, introduced by the removal of these glands. In the isolated tissues, under the conditions I have described, complete resumption of carbohydrate oxidation may be simpler to achieve, since it is not dependent on a complicated organization, and merely has to maintain respiration.

Dr. Burk: In the last few slides you showed, I noticed that the oxygen consumption did not change during the 10-hour run, but that it was really the R.Q. that rose; that means that mainly CO_2 is increasing. Do you have a more detailed picture of what is happening? Is it, first, that the substrate is changing, or is it the nature of the processes changing?

Dr. Shorr: In the absence of chemical studies showing the disappearance of carbohydrate in amounts equivalent to the oxygen uptake and the level of the respiratory quotient, one cannot be certain that a rising CO_2 production is not the result of a non-oxidative process, such as decarboxylation. We shall try to obtain this necessary type of information. However, the former assumption is generally made in *in vitro* studies when the addition of glucose or lactate, for example, results in a rise in the respiratory quotient. In some instances, as in the work on mammalian tissues poisoned with iodoacetic acid, Barker and I were able to show a corresponding disappearance of glucose. While it is usual for the addition of glucose to cause a rise in oxygen consumption, as well as an elevation of the respiratory quotient, this is not always the case. In excised cardiac muscle, glucose elevates the respiratory quotient without increasing respiration. Some support of the view that the increase in CO_2 production in our experiments does signify increased carbohydrate oxidation is derived from the fact that the respiratory quotient rises to unity and remains there, going no higher. If the CO_2 had a non-oxidative origin, the respiratory quotient would presumably continue to rise above unity.

Dr. Burk: I take it you would consider in this explanation that probably during all this time the oxidizing mechanisms are saturated and this accounts for the constant Q_{O_2}.

Dr. Shorr: I do not think that this is a necessary assumption. The level of respiration in the presence of glucose is not the maximum of which excised cardiac tissue is capable with other substrates. It is characteristic of this tissue that the respiratory quotient rises in the presence of glucose without there being any rise in the oxygen consumption. Lactate, on the other hand, raises both the respiration and the respiratory quotient. Since the substrate used in all the prolonged experiments with cardiac tissue was glucose, we would not expect that the oxygen consumption would increase, but that the carbon dioxide formation would rise.

Dr. Hogness: We have recently made some analyses of various mammalian tissues for cytochrome-c, and find that in skeletal muscle the amount is about 6 gamma per gram, in cardiac muscle about 18 or 19 gamma per gram, and in pigeon breast muscle approximately 50 gamma per gram. In embryonic tissue and in cancer tissue it is practically zero. But the interesting fact is

that it is usually present in high concentrations in those tissues which show a high aerobic glycolysis. This suggests that perhaps there is a connection between the dehydrogenation of lactic acid and cytochrome-c. Can you tell me which particular step in the respiratory chain is blocked in the tissue of the diabetic?

Dr. Shorr: We do not know where the specific block occurs in diabetes. There does not appear to be any disturbance in the over-all respiration of the cell. Indeed, the diabetic organism has a higher metabolic rate than the normal animal and excised cardiac tissue from the diabetic dog has a somewhat higher rate of respiration than that from normal animals. I suspect that we shall have to look elsewhere than to the respiratory ferment and cytochrome system for the site of the defect. After our early work with diabetic skeletal muscle, which we found lost the capacity to resynthesize added lactate, we were inclined to consider that this defect, which explained the poor storage of glycogen in the diabetic organism, might represent the initial block. When we carried out similar experiments with cardiac muscle from depancreatized dogs, we were surprised to see that this tissue could resynthesize lactic acid, but could not oxidize either glucose or lactic acid. The lactic acid enzyme system in this tissue appears to function independently of insulin. The one metabolic defect both skeletal and cardiac tissue had in common in diabetes was in the oxidative mechanism for dealing with carbohydrates.

With regard to your statement that cytochrome-c is usually present in high concentrations in tissues with a high aerobic glycolysis, cardiac muscle would appear to be an exception. Its cytochrome-c content is high, as you have shown, but it has no aerobic glycolysis.

Dr. Cori: I wonder whether the changes in lactate would be large enough to demonstrate an increase in glycogen. Have you made any attempts to measure glycogen to determine the fate of the disappearing lactate?

Dr. Shorr: The changes in lactate with cardiac muscle particularly are sufficiently large to permit of reliable chemical balances. It is our intention to carry out the type of experiment you suggest.

Dr. Burk: With regard to the effect of insulin, you calculated your data in terms of rates. Would you come to your same conclusion with regard to all the insulin data we have had presented at this Symposium, especially in the paper of Stare and Baumann, namely, that insulin has an effect on the rate just at the very end?

Dr. Shorr: You are referring now to the data presented at this Symposium by Stare and Baumann and by ourselves on the *in vitro* effect of insulin on the respiration of pigeon breast mince. In our own experiments, the effect of insulin on the oxygen consumption was detectable only after respiration had fallen to a fraction of its initial value, generally 50 p.c. or less. In no instance could insulin be considered to have maintained respiration at anywhere near the original level. Their experiments, and those of Barker and myself, agreed in this respect.

Dr. Barker: Stare and Baumann suggested that the effect of insulin on the respiration of minced mammalian muscle might be demonstrated better in the presence of saline added to the phosphate. We have done some experiments using this type of solution and found the same lack of effect of the insulin as with Krebs' solutions.

Dr. Warren: What is the effect of iodoacetic acid on aerobic glycolysis? Does it exert an effect in the concentrations at which you used it in the aerobic experiments to demonstrate that carbohydrate is oxidized in the presence of iodoacetic acid?

Dr. Shorr: Crabtree and Cramer, working with tumor tissue, were able to show that iodoacetic acid caused as prompt and complete inhibition of aerobic as of anaerobic glycolysis.

Dr. Lipmann: What do you think about the non-inhibition of respiration with iodoacetic acid? Do you think it shows the necessity of another pathway of glucose oxidation, suggested by the work of Warburg and myself and Dickens on oxidation of gluconic acid phosphate?

Dr. Shorr: It would seem necessary to infer from our experiments that another pathway for glucose oxidation exists which is utilized by the tissues when poisoned by iodoacetic acid. Just which substrate may be oxidized still remains a matter of speculation. The work you cite makes gluconic acid phosphate a possibility. It should be pointed out in this connection that it is difficult to evaluate much of the work with iodoacetic acid, particularly that done with tissue extracts, because of the enormous difference in the concentrations used in these experiments and those we reported today. We used the minimum concentration which would completely abolish glycolysis. Most of the tissue extract work was done with far greater concentrations. Our ideas as to which processes are inhibited by iodoacetic acid are almost entirely based on these latter experiments. There is no certainty that these inhibitions would exist under the conditions of our experiments. Indeed, there is already evidence of a discrepancy in the behavior of glucose under these two experimental set-ups. The phosphorylation of glucose is prevented by iodoacetic acid in high concentration in tissue extracts. Phosphorylation is assumed to be a necessary preliminary to glucose

oxidation. Yet isolated tissue slices, under our experimental conditions, were able to oxidize added glucose in the presence of concentrations of iodoacetic acid which completely abolished glycolysis. Such divergent results make it imperative to reinvestigate the whole iodoacetic acid problem under conditions which will make for specificity and avoid concentrations which might introduce nonspecific factors. Until that is done, we shall have to postpone judgment as to the nature of the carbohydrate oxidation taking place in iodoacetic acid poisoned tissues.

Dr. Cori: The phosphorylation of glycogen is not inhibited even by very high concentrations of iodoacetic acid, so that if a tissue contains glycogen, hexosemonophosphate can still be formed. The phosphorylation of glucose, on the other hand, is inhibited by iodoacetate.

Dr. Shorr: Cori's observations on hexosemonophosphate formation in the presence of iodoacetic acid are of great interest in that they point out an important carbohydrate intermediary which might be available for oxidation in iodoacetic acid poisoned tissue. In what concentrations of iodoacetic acid is the phosphorylation of glucose inhibited?

Dr. Cori: That is difficult to say, because some iodoacetic acid is removed by oxidation. That seems to be one difficulty when low concentrations of iodoacetic acid are used.

Dr. Shorr: The question is that of the possibility of enough oxidation of iodoacetic acid, when low concentrations are used, to permit the tissue to escape from its influence. Since oxidation would not occur under anaerobic conditions, we might wrongly assume that the same complete inhibition of glycolysis we were observing under anaerobic conditions was existing in the tissues studied in oxygen. I think the concentrations used and the proportion of solution to mass of tissue is such as to make this a little unlikely. They were approximately the same concentrations as those used by Cori and Cori in their work on frog muscle. The work of Crabtree and Cramer on tumor tissue, using much the same concentrations of iodoacetic acid, showed a persistence of inhibition of aerobic glycolysis, indicating that the oxidation of iodoacetic acid was insufficient to permit the escape of the tissue from its influence.

Dr. Cori: That might be possible. But as I understand the issue, the question is whether or not the iodoacetic acid concentrations used would permit phosphorylation of glucose. When glucose is phosphorylated, hexosemono- and hexosediphosphates are formed.

Dr. Barker: Would it not also be possible to have the glucose condensed to glycogen directly, phosphorylated, and broken down from that point?

Dr. Cori: I think phosphorylation would have to take place first. So the same considerations would hold.

Dr. Shorr: Since glucose was oxidized in our experiments with iodoacetic acid, may we not have to assume, for the present, that it undergoes phosphorylation in the presence of iodoacetic acid concentrations sufficient to abolish glycolysis? Otherwise we shall have to postulate a direct oxidation of glucose under these conditions.

Dr. Fleischmann: I would like to ask Shorr whether this independence which he mentioned in tissue respiration *in vitro* has anything to do with the old observation that the respiration of liver tissue of small animals and of large animals *in vitro* is very similar, and has nothing to do with the great differences which we must expect from their B.M.R. Some of these observations were made some years ago by Grafe and by Terroine. In some observations of my own, I took tissues out of hibernating animals and waking animals of the same species and examined their respiration and always found the Q_{O_2} depended merely on the temperature of the Warburg system and was quite independent of whether I took that tissue out of sleeping or waking animals. I did not run any R.Q.'s on these, but I feel they might contribute to this problem.

Dr. Shorr: The experiments you mention bring additional support to the view that tissues in the living organism are subject to the control of a variety of factors influencing the rates as well as the character of their reactions. On removal from the body, the tissues are released, at least in part, from these influences. I am inclined to favor this view, in the light of some of the experimental data I have reported today.

Mr. MacLeod: There is a rather striking instance of the selective action of an inhibitor on aerobic and anaerobic glycolysis. The human sperm has a quite unique metabolism, for it has a very small respiration and a very high aerobic glycolysis, the aerobic glycolysis being just about 85 per cent of the anaerobic. At one time I tried to determine the effect of methylene blue on the respiration and I found that methylene blue in oxygen was definitely very toxic to the sperm. So then I tried the effect of methylene blue on the glycolysis, merely to see whether the aerobic glycolysis would be affected, and I found that very small quantities of methylene blue (0.5 mg. p.c.) will stop aerobic glycolysis within half an hour; motility stops with the glycolysis, while anaerobically you can use up to forty times the amount of methylene blue and get no effect whatsoever on anaerobic glycolysis or upon the motility of the cell in N_2. This would seem to be definitely related to a permeability factor, although I have no

way yet of showing whether the methylene blue penetrates the cell anaerobically; I would suspect it did not.

Dr. Stern: Concerning MacLeod's observation as to the effect of methylene blue on sperm, I do not think this is such an isolated observation. Friedheim, for instance, has found that pyocyanin, a dyestuff similar to methylene blue in E'_o, will decrease the aerobic glycolysis of tumor tissue.

Mr. MacLeod: I have observed that pyocyanin is much more toxic than methylene blue in the case of sperm. Whether or not methylene blue inhibits the aerobic glycolysis of tumor tissue, I would say that permeability is a factor in this case also.

Dr. Cori: I would like to ask Shorr a question concerning the escape phenomenon which he observed on the muscles of depancreatized animals, namely, whether they also regain the capacity to glycolyze. As I understand it, there is a deficiency in the glycolytic capacity of skeletal muscle from depancreatized dogs.

Dr. Shorr: Perhaps I should have made it clearer in my paper that most of the experiments on the "escape phenomenon" were carried out with cardiac tissue from diabetic animals. This tissue, in contrast with skeletal muscle, preserves its capacity to glycolyze in the absence of insulin. Hence it is not a suitable tissue to use to get an answer to the question you have raised. This much can be said, that it preserves its ability to glycolyze under these conditions. By varying buffer and substrate, the terminal rate of glycolysis can be influenced without interfering with the recovery of carbohydrate oxidation. It is for that reason that I would consider the defect in diabetes to lie in the oxidative rather than the glycolytic mechanism. Skeletal muscle is ideally fitted to answer this question. Up to now relatively few experiments have been carried out with this tissue. Restoration of carbohydrate metabolism was found to occur. Unfortunately studies of the glycolytic mechanism with skeletal muscle under these conditions have not as yet been made.

Dr. Chambers: If data from the whole animal can be applied to the question of glycolysis, it is interesting to note that when depancreatized dogs were exercised on a treadmill, the rise in blood lactic acid was somewhat larger than in the normal animal. The recovery process to a basal level of blood lactate was slower in the diabetic animal.

Dr. Shorr: It is always gratifying to obtain support for *in vitro* data from experiments on whole animals. The important body of knowledge acquired by the study of the intact organism is all too often disregarded by workers in the field of intermediary metabolism. I should like to call attention to the bearing of Chambers' observation on some of the material I reported today. It was found that diabetic skeletal muscle glycolyzed poorly at rest, and did not resynthesize *added* lactate. From this evidence, it might have been inferred that the diabetic dog is able to form lactic acid readily on exercise, and this lactic acid eventually disappears, though more slowly than in the normal animal. Furthermore, Cori and Cori and others have shown that most of the resynthesis of lactic acid does not take place in the muscles but in the liver. The *in vitro* experiments therefore should be reinterpreted on the basis of the results obtained with the whole organism. What can be said is that they demonstrate a definite defect in the glycolytic mechanism in diabetic skeletal muscle. Its reserve is lower. It cannot handle the extra load of added lactate which the normal muscle can manage successfully. The low glycolysis under resting anaerobic conditions, and the failure of added glucose to increase anaerobic glycolysis point also to a lessened reserve of the lactic acid enzyme system as compared to normal muscle. Nevertheless under stimulation (exercise) the working muscle in the living diabetic animal can form considerable amounts of lactic acid. The significance of the failure of resynthesis of diabetic muscle *in vitro* for the living animal is tempered by the demonstration that the largest part of the resynthesis does not take place directly in the muscle.

The *in vitro* work is of prime importance in demonstrating mechanisms which may potentially participate in the economy of the whole organism. The actual part played is best determined by an evaluation of evidence obtained on a variety of experimental levels. Often the living organism is the court of last resort.

Dr. Warren: It is interesting to recall that in blood there is no defect in the glycolytic mechanism in diabetes such as you have demonstrated in the case of skeletal muscle. This means that this chemical abnormality is not generalized as, indeed, you have already pointed out in the case of cardiac muscle.

RESPIRATORY ENZYME SYSTEMS IN SYMBIOTIC NITROGEN FIXATION*

R. H. BURRIS AND P. W. WILSON

In many fields of biology enzyme research falls into two general classes. First, there are studies which are primarily concerned with enzymes as such, their occurrence, distribution and properties. In these studies it is desirable to obtain as pure a preparation of the enzyme as possible and then to reconstruct the system and study its characteristics *in vitro*. The scientific worker interested in this phase of enzyme research does not usually study any one particular group of organisms or tissues but investigates many groups so as to determine the properties of the same enzyme as it occurs in different environments.

Second, there are studies in which the chief interest lies not so much in the occurrence and properties of the enzyme *per se* but in its metabolic significance for the organism. In these studies isolation of the enzyme from the cell may not always be desirable since the object is to determine the characteristics of the system as it functions in the complex, heterogeneous environment of the living organism rather than in the somewhat artificially simple environment presented by the reconstructed system in the test-tube. Research workers engaged in such studies are usually interested primarily in a particular group of organisms or tissues whose biochemistry is under investigation.

It should be emphasized that the two types of research are not to be regarded as opposing schools of thought with respect to the proper manner of carrying out research on chemistry of enzymes, but are actually supplementary investigations, both of which are necessary for a complete understanding of the complicated problems of this field. In the initial studies on a given enzyme system the first type of research is ordinarily followed since it is usually necessary to make some sort of isolation from the complexities of the intact cell before interpretation of the experimental observations becomes possible. Following the survey of the occurrence and properties of a given enzyme in various species, investigations are undertaken to apply the methods developed and findings made to the problem of how the metabolism of a specific organism reflects the activity of the enzyme under investigation.

A number of the papers in this Symposium will represent primarily the first type of research. This paper presents an example of the second, and illustrates how the methods developed and the information gained in the more theoretical studies

on respiratory enzymes have been applied to some of the complex questions still to be answered in the field of symbiotic nitrogen fixation, *i.e.*, fixation through the association of leguminous plants and bacteria.

Although research on symbiotic nitrogen fixation observed its centennial last year, an understanding of the basic chemistry and physics of the process is still lacking. As has been discussed by Wilson (1937, 1939), the following questions concerned with the mechanism of invasion of the plant and the fixation of nitrogen through the association await investigation:

1. What is the biochemical explanation of the specificity[1] in the cross-inoculation groups? The organisms belonging to the various species of *Rhizobium* are so very similar in their morphological and physiological characteristics that ordinarily they would not be classed as separate species. About the only outstanding difference is their rather high degree of specificity in the invasion of the host plant.

2. What is the cause of strain variation among species of the root nodule bacteria? This question is an extremely important one economically since strain variation especially in relation to host plant specificity must be carefully controlled in the commercial distribution of the bacteria for artificial inoculation of legume crops. As yet, there is no way to determine whether a given strain will fix nitrogen with a given host plant except by greenhouse or field test. The different strains are identical as far as the usual morphological and physiological characteristics are concerned.

3. Which is the active agent of fixation—the bacteria, the plant or both? Attempts to secure fixation by either component in isolation have met with little consistent success. Occasionally positive reports appear with respect to fixation by the bacteria on laboratory medium, and more rarely, the plants in absence of the bacteria are alleged to use free nitrogen, but repetition of the supposedly positive findings by most workers has met with failure.

4. Finally, the chemical mechanism of the fixation process is yet unknown. Although several suggestive leads have been lately uncovered, the pathway by which the inert nitrogen molecule of the air

[1] The root nodule bacteria (*Rhizobium sp.*) are divided into *species* according to the host plants with which they associate. The genera of plants on which nodules are formed by a given species of bacteria constitute a *cross-inoculation* group. Each isolation of a given species of the bacteria is regarded as a pure-line *strain*. Some strains of a given species will fix large quantities of nitrogen in association with the proper host plant, whereas others fix little or none although good nodulation is secured. Variation in the ability of strains to fix nitrogen in association with the host is not entirely a function of the bacteria but also depends on the genus, species or even variety of the plant. A strain efficient in the fixation of nitrogen on one species of host plant, may be poor on another. This type of variation is called *host plant specificity* (Fred, Baldwin and McCoy, 1932; Wilson, 1937).

is converted into living tissue remains more a matter of speculation than a matter for definite, well-supported statements.

Attack on the foregoing problems has been largely in the hands of those primarily interested in answering localized questions. As a result a great deal of the investigation has been more in the form of "trouble shooting" rather than fundamental research. Of late it has become increasingly evident that empirical attack on these questions has reached the point of diminishing returns, and that the applied phases of symbiotic nitrogen fixation would be greatly benefitted if a clearer understanding of the biochemistry of the process were available. At the University of Wisconsin an attempt has been made to solve some of the biochemical problems through application of the methods and points of view which have been developed in the research on respiratory enzymes. Such application has been made both to the bacteria in isolation and to the intact system of plant and bacteria (Wilson, 1939). This paper will be primarily concerned with the results of research which seeks to answer the following specific questions:

1. Is the respiratory system of the root nodule bacteria similar to that found in other microorganisms and tissues, or is the function of nitrogen fixation uniquely associated with an unusual type of respiration?
2. Are there fundamental differences in the respiratory systems of the different species or strains of the root nodule bacteria which might be correlated with the observed specific differences in their ability to fix nitrogen in association with the various legumes?
3. Do differences exist in the respiratory system of a given strain when grown on laboratory medi-

plied by Wilson (1938) have been used, but a modified Thunberg technique has been developed which consists in the use of a large Thunberg tube designed to fit the Evelyn photoelectric colorimeter. Through use of a 540 millimicron filter, the methylene blue concentration can be determined at any point in the reduction process, and thus the kinetics of the reduction are established rather than merely the point of 90 per cent reduction (Tam and Wilson, 1938).

In most cases a straight line is obtained by plotting the log of the methylene blue concentration against time. If there be any variation, it appears as a break in the line and suggests that a change may have occurred in the activity of the dehydrogenase system. For example, the accumulation of metabolites may cause such a break.

Comparison of Strains and Species of Rhizobia

Previous attempts at differentiation among strains and species of the root nodule bacteria have been based on characteristics of growing cells. Results of such investigations are difficult to analyze, and it would be preferable to state the similarities and differences among the organisms quantitatively in terms of the properties of certain enzyme systems as, "succinate is oxidized three times as rapidly as is glucose", rather than the indefinite characterization covered by such observations as, "growth occurs on both glucose and succinate". Recently, we have completed a survey of the effect of various environmental changes, such as temperature, pH and pO_2 on the respiratory enzyme systems of "resting" cells representing 10 strains and 5 species of the root nodule bacteria. These were:

	Species	"Good Strain"	"Poor Strain"	Cross-inoculation group
"Fast[2] growers"	R. meliloti	100	101 (variable), C_1	Alfalfa-sweet clover
	R. trifolii	209	202	Clover
	R. leguminosarum	317	333	Pea - vetch
"Slow[2] growers"	R. japonicum	505	507	Soybean
	Rhizobium sp.	602	—	Cowpea

um in comparison with growth in association with the plant? This question is of basic significance for the entire field of symbiotic nitrogen fixation since most of the available information on the properties of the organisms has been secured with laboratory cultures, whereas the function of chief interest is exercised only in association with the host plant.

Methods

The general method employed in these studies has been measuring under various conditions the respiratory activity of "resting cells" of the bacteria. The usual Warburg techniques as ap-

pH.

It is customary to culture the rhizobia at a pH near 7.0. Studies were made of the effect of pH on the rate of respiration of washed suspensions of rhizobia, using phosphate, borate and phthalate buffers. The initial and final pH were determined with a glass electrode and vacuum tube potentiometer, and the average value used. The pH-rate of respiration curves have been reproduced in the

[2] Refers to their apparent rate of growth on a standard yeast-water mannitol-agar medium.

papers by Wilson (1938, 1939). In general, those for the strains of the fast growers differ only in minor details, all possessing a rather broad optimum in the neighborhood of pH 6.5.

The strains of *R. japonicum* (soybean) exhibit a quite different response. The pH optima are distinctly higher than for the fast-growing organisms, 505 respiring best at pH 8.65, and 507 showing its optimum at 8.15. The curves for this species are further distinguished by a plateau region occurring in the pH range optimum for the fast-growers, roughly pH 5.5 to 7.5.

In every case, except *R. meliloti* 100, the limiting pH, where the respiration is zero, is between pH 4 and 5 on the acid side and between 9 and 10 on the alkaline side. The lower limit for *R. meliloti* 100 is pH 5.2.

The maximum rate of reduction of methylene blue by the fast-growing rhizobia occurs at a higher pH than does the maximum rate of oxygen uptake of the same strain. In our laboratory Tam (1939) has found that the range pH 8.0 to 8.2 is optimum for methylene blue reduction by *R. trifolii* 209 on succinate, glucose, lactate and formate. As in the case of oxygen uptake, the limiting pH is near 5 on the acid side and between 9 and 10 on the alkaline side.

Generalizing, we can say that the fast-growing rhizobia show no appreciable differences between species or between good and poor strains in their pH response. The slow-growers have a higher optimum pH and show a plateau in the region optimal for the respiration of the fast-growers. Methylene blue reduction is accomplished most rapidly by the fast-growers at a pH of about 8.

Temperature

Temperature studies on the respiration of the rhizobia were made with glucose as a substrate, at a pH of 6.0. Wilson (1938) discusses a typical temperature-rate of respiration curve which was obtained with *R. trifolii* 209. The optimum falls at about 37.5° C. Above this point, inactivation is rapid. The response of all strains examined was very similar to that of *R. trifolii* 209, their optimum temperatures falling within a degree of 37.5° C.

The slope of the line, obtained by plotting the reciprocal of the absolute temperature against the log of the rate of reaction, when multiplied by 4.6 gram calories gives the μ value, or temperature characteristic of a biological reaction. When temperature data were plotted in this manner for the respiration of glucose by 6 strains of rhizobia representing species from alfalfa, clover, pea and soybean, and when the lines were fitted to the experimental points by the method of least squares, the following μ values were obtained:

R. meliloti 101	12.0 kg.-cal.
R. trifolii 202	8.9
R. leguminosarum 317	10.4
R. leguminosarum 333	9.1
R. japonicum 505	10.7
R. japonicum 507	7.5

Statistical analysis of the data used for the determination of these mu values indicates that only *R. japonicum* 507, and possibly *R. trifolii* 202, possess a temperature characteristic which is significantly different from those of the other strains. Most of the values clustered about 10,000 to 11,000 calories.

Crozier (1924) has studied the effect of temperature on a large number of biological reactions and has often noted a break in the curves for estimation of μ values at about 17° C., finding that below this temperature the values of μ increased greatly. The response of two strains of the root nodule bacteria at 5.2°, 7.8°, 10°, 12.5° and 15° has been determined; the μ values were estimated as 24.3 kg.-cal. for *R. trifolii* 209 and 21.6 for *R. leguminosarum* 317. These values are more than double those found in the normal temperature range for the same organisms.

Crozier attaches considerable significance to this break in the lines. Belehradek (1935), however, does not believe that the change in the μ value has any significance and attributes the break in the slope of the line to the fact that the method used to evaluate μ does not represent any fundamental relationship between temperature and biological activity. He proposes an alternate empirical expression in which the log of the temperature in degrees centigrade is plotted against the log of the reciprocal of the velocity. For comparative purposes the slope of this line fulfills the same function as does a μ value. By plotting the data for *R. trifolii* 209 and *R. leguminosarum* 317 in this manner, a straight line is obtained from 5.2° to 37° with no break at 17° (Fig. 1). In the normal temperature range the straight line from such plotting does not fit the data any more closely than does that of the μ curve, but the advantage in the extended range is obvious. Statistical analysis indicated that the slopes of the two lines in Fig. 1 did not differ significantly.

Tam (1939) has studied the effect of temperature on methylene blue reduction with the rhizobia, using glucose as a substrate. He found that the optimum temperature of reduction for *R. trifolii* 209 is 42°, for *R. trifolii* 202, 46°, for *R. leguminosarum* 317, 45°, and for *R. leguminosarum* 333, 43° C. All the values fall near 45° C., without particular differences between species or strains. Inactivation above the optimum temperature is very rapid. It is evident that the temperature for
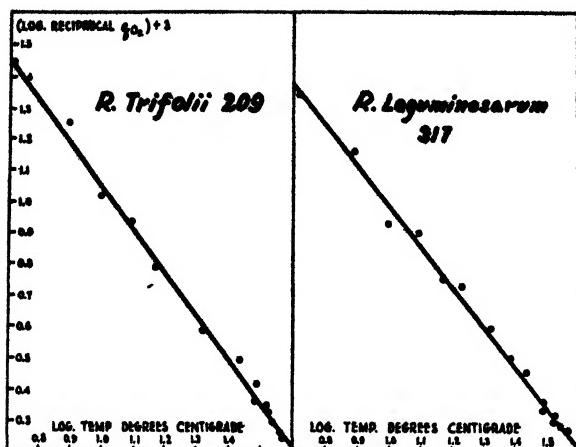
Fig. 1. Comparison of slopes of lines showing relation between temperature and rate of respiration plotted according to Belehradek (1935).

the maximum rate of anaerobic dehydrogenation is distinctly higher than that for the most rapid uptake of oxygen by the organisms.

Respiratory Enzyme Systems of Organisms in vitro and in vivo

As suggested earlier, a fundamental question in symbiotic nitrogen fixation is whether or not the biochemistry of the organisms grown on a laboratory medium is similar to that of the organisms growing in association with the host plant. Most studies on these organisms have been restricted to laboratory cultures, since plant structures, such as nodules, are unsuitable for use in long-time growth experiments. As a result the biochemistry of the root nodule organisms as they exist in association with the host has not been investigated. Thorne and Burris (1938) have developed a method based on the use of a glass homogenizer which makes it possible to secure suspensions of the organisms directly from the nodules and thus to compare the characteristics of enzyme systems possessed by bacteria grown in association with the plant with those of organisms grown on a laboratory medium. Very little debris remains in the suspensions of organisms taken directly from the nodule, so that the determinations are made on the bacteria alone and are not complicated by the presence of plant tissue. The organisms taken from the nodule will be termed *nodular* bacteria and those from laboratory culture, *cultured* organisms. Comparisons have been made between the respiratory systems possessed by these two groups of bacteria with respect to their response to changes in pO$_2$, type of substrate, and presence of specific inhibitors. This work may have significance beyond that of the immediate problem, for it presents a unique opportunity to study the

metabolism of an organism which has grown in association with its host. In general, investigators have had to be content with information about parasitic and symbiotic organisms as they grow in the laboratory rather than in their natural habitat.

Effect of the pO$_2$.

The effect of oxygen tension on 8 strains (4 species) of the rhizobia has been investigated using glucose as the substrate at a pH of 6.5 Air was displaced from the Warburg flasks with gas mixtures containing a known pO$_2$; nitrogen and hydrogen have been used interchangeably as the inert gas.

In Fig. 2 and 3 the oxygen tension is plotted on the abscissa and per cent of respiration in air on the ordinate; that is, the rate of respiration in air is taken as 100. The fast-growing organisms, alfalfa 100, clover 202 and 209, and pea 317 and 333, show very similar curves. They require about 0.1 to 0.15 of an atmosphere of oxygen to respire at their maximum rate. The typical slow-
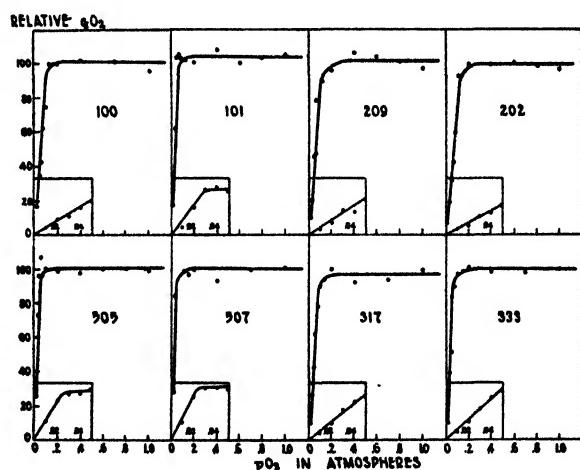
Fig. 2. Comparison of the pO$_2$ functions of different strains of "cultured" rhizobia.
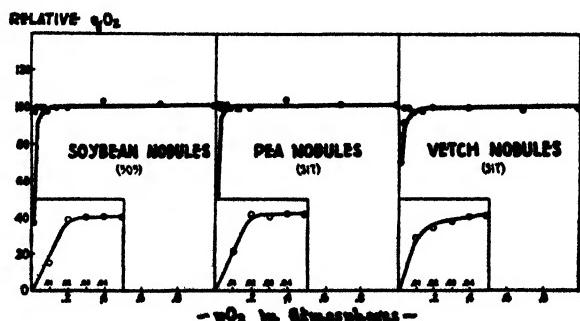
Fig. 3. The pO$_2$ functions of "nodular" suspensions of rhizobia.

TABLE I

Relative rates of respiration on different substrates by strains of rhizobia.

Substrate	Strain of Organism									
	100	101	C-1	202	209	317	333	505	507	602
Glucose	100	100	100	100	100	100	100	100	100	100
Endogenous	20	29	30	14	16	16	14	46	39	43
Arabinose	40	47	52	78	83	78	65	126	123	137
Xylose	95	82	60	66	65	69	61	102	118	123
Galactose	77	93	57	70	83	65	66	104	114	77
Levulose	34	47	53	68	76	59	56	104	90	62
Mannose	70	69	78	45	86	92	92	62	104	93
Sorbose	48	58	59	39	64	69	54	133	44	58
Sucrose	76	80	87	83	84	87	87	82	83	78
Rhamnose	41	42	55	77	66	67	59	169	143	188
Raffinose	15	18	26	14	21	27	28	62	73	25
Maltose	79	95	98	69	74	74	66	104	109	74
Lactose	52	54	80	81	74	80	76	67	85	42
Cellobiose	52	56	97	74	78	86	75	150	78	57
Trehalose	30	48	92	38	86	73	73	83	75	77
Ethylene glycol		26	43						56	
Glycerol	33	37	85	36	66	52	46	110	98	84
Erythritol	23	34	62	23	54	39	31	65	53	75
Ethyl alcohol	36	26	51	14	20	27	14	102	88	209
Mannitol	27	27	40	21	46	30	35	58	59	109
Sorbitol	39	46	59	27	71	28	30	133	64	92
Dulcitol	30	33	43	12	24	21	23	75	47	81
Inositol	42	40	92	31	73	57	59	83	27	76
Na formate	40	37	62	40	33	28	15	106	105	403
Na acetate	84	76	126	45	41	43	38	208	117	660
Na pyruvate	60	67	104	67	75	70	53	308	279	643
Na lactate	62	93	83	67	72	75	68	251	263	399
Na oxalate	27	51	41	16	18	12	17	81	100	280
Na malonate	31	44	44	24	23	25	21	200	158	407
Na maleate	49	85	86	31	31	35	38	166	200	400
Na succinate	118	113	137	129	129	116	99	359	455	1006
Na malate	64	67	169	51	108	79	69	350	305	314
Na fumarate	99	87	106	123	112	99	84	261	205	494
Na oxalacetate	83	80	109	43	89	78	64	361	379	503
Na tartrate	16	33	42	25	16	19	14	92	95	125
Na citrate	24	56	47	16	21	15	16	193	98	263
qO_2 on glucose	454	384	264	521	461	442	460	132	156	192

Values given expressed as percentage glucose respiration, *i.e.*, glucose equals 100.

Warburg flasks contain 1 ml. pH 6.5 M/15 phosphate buffer, 1 ml. M/50 neutral substrate, 1 ml. bacterial suspension, and 0.15 ml. 20 p.c. KOH in inset cup.

qO_2: mm.^{3} O_2 uptake per hr. per mg. cell nitrogen.

growing organisms, soybean 505 and 507, attain their maximum rate of respiration at much lower oxygen tensions, approximately 0.03 to 0.04 atmosphere of oxygen. Alfalfa 101 responds like the slow-growers; though it is considered a fast-grower, its growth rate is actually intermediate between the two groups. If the insets of Fig. 2 which cover the pO_2 range from 0.0 to 0.05 atmosphere are examined, the differential response of the fast and slow-growers to changes in the pO_2 is more evident. Cultures 101, 505 and 507 rise rapidly to their maximum rates, whereas the other organisms are well below their maximum rate of respiration even at a pO_2 of 0.05 atmosphere.

Since the oxygen tension within an actively metabolizing nodule is lower than that at an agar surface, an adaption in the oxygen requirements of cultured and nodular organisms might be expected. Comparison of the data in Fig. 2 and 3 suggests that such is actually the case. Fig. 3 shows that, independent of the species, organisms grown in the nodule reach their maximum rate of respiration at low oxygen tensions. The curves are very similar to those for the pure cultures of the slow-growers. The insets show that the nodular organisms from pea and vetch plants inoculated with *R. leguminosarum* 317, respired at near maximum rates at 0.03 atmosphere of oxygen, whereas, as shown in Fig. 2, a pO_2 of 0.15 atmosphere was required for comparable respiration by the cultured *R. leguminosarum* 317.

Effect of substrate

Observations of the relative rates of oxygen uptake on different substrates often suggest the types of enzyme systems which are active in a given tissue.

The root nodule organisms, which are typical of the less fastidious types of bacteria, existing under wide extremes of environment and available food supply, attack a variety of substrates. The rate of respiration of 10 strains of cultured rhizobia has been determined on 35 substrates (Table I), and nodular organisms have been tested on a smaller number (Thorne and Burris, 1938).

Glucose serves as an excellent substrate for the fast-growing rhizobia, and for them it is superior to all the other sugars, all the polyhydric alcohols and most of the organic acids tested. There is no striking difference in the utilization of pentoses, hexoses, or di- and polysaccharides by the fast-growers. On the other hand, glucose is inferior to many substrates for the slow-growers, this group favoring organic acids as the source of carbon. Except for certain cases among the slow-growers, the polyhydric alcohols are poor substrates. Succinate appears to be the best substrate employed, and the other four carbon dicarboxylic acids are also good. The three carbon organic acids, lactic and pyruvic acids, likewise support rapid respiration by the slow-growers.

In Table II are summarized typical data from the paper of Thorne and Burris (1938). These show that the rates of substrate oxidation by the nodular and cultured organisms differ only slightly. The apparently greater respiratory rate of the nodular organisms on the poor substrates usually can be accounted for on the basis of their higher endogenous respiration, though this explanation hardly suffices for the improvement in the utilization of mannitol by the organisms from vetch nodules compared with *R. leguminosarum* 317. Cultured and nodular organisms of the fast-growing types respired about the same on lactate,

TABLE II

Relative rates of respiration on various substrates by "nodular" and "cultured" rhizobia.

Treatment	Pea Organism "Fast Grower"			Soybean Organism "Slow Grower"	
	317 Culture	Pea Nodules	Vetch Nodules	505 Culture	Soybean Nodules
Glucose	100	100	100	100	100
Endogenous	16	54	40	46	89
Arabinose	78	74	90	126	100
Mannitol	30	62	100	58	85
Lactate	75	68	85	251	168
Succinate	116	155	165	359	308
qO_2 on Glucose	442	132	182	132	135

whereas the cultured slow-growing organisms did much better on this substrate than did the nodular organisms. On succinate the bacteria from pea and vetch nodules respired at a distinctly higher rate than did cultured *R. leguminosarum* 317. The reverse is true with soybean nodule organisms and *R. japonicum* 505.

In summary, it appears that the same enzyme systems exist in the nodular and cultured organisms, but there may be a somewhat different balance in the intensity of their action. Perhaps again, there is an adaptation by the bacteria within the nodule in response to the materials supplied by the plant. The differences, however, are less striking than are the similarities between the organisms from the two sources.

Effect of inhibitors

Since their action is frequently quite specific, inhibitors often prove valuable in establishing the presence of particular enzyme systems. Some inhibitors depend upon the alteration of the colloidal properties of the enzyme, and the action in such cases may be quite different in the intact cell than with the isolated enzyme preparations. As most of the work with inhibitors has been with animal tissue, we must depend largely upon analogy in interpreting results with bacteria.

The effects of a number of inhibitors on the metabolism of glucose and succinate by nodular and cultured organisms have been determined. In all cases the bacterial suspension and the neutral inhibitor were mixed and incubated at room temperature for a short time before the addition of the substrate, since, at times, the substrate may exert a protective action if it combines with the enzyme before the addition of the inhibitor. The results with the cultured organisms are given in Table III. As Thorne and Burris (1938) have presented the corresponding data for nodular suspensions, these need not be repeated. The general conclusions from all the studies, however, will be summarized.

1. *Oxidase inhibitors.* Cyanide is a strong inhibitor of oxidases and is believed to block the cytochrome system by virtue of its inhibition of cytochrome oxidase. We have observed typical cytochrome bands in heavy suspensions of rhizobia examined with the microspectroscope, so an inhibition of respiration by cyanide might well be expected. M/1000 cyanide usually gives over 90 per cent inhibition of oxygen uptake by nodular and cultured organisms on either succinate or glucose. M/10,000 cyanide is strongly inhibitory, and in some cases inhibition is still evident at a M/100,000 concentration. The effect on succinate is usually somewhat greater than that on glucose. The influence on comparable cultured and nodular suspensions is almost identical.

The effect of sodium azide is usually considered to be similar to that of cyanide, but this does not entirely hold true with these organisms. With *R. leguminosarum* the cultured organisms were inhibited more than the nodular organisms, and the respiration with succinate as a substrate was decreased more than with glucose. Moreover, when glucose was used, quite unexpectedly, *stimulation* was noted with vetch nodule preparations and azide in all concentrations used and with pea nodule suspensions and azide in the M/1000 concentration. The corresponding cultured organisms were inhibited under the same conditions. The nodular preparations of the soybean and cowpea organisms were inhibited more than the cultured suspensions.

In concentrations of M/100, the Hecht-Eicholtz reagents (1-amino 8-naphthol 4 sulfonic acid and *o*-aminophenol-*p*-sulfonic acid), which are considered specific for heavy metal catalysis, were weakly inhibitory and more commonly stimulatory.

2. *General dehydrogenase inhibitors.* Urethane is typical of the indifferent narcotic inhibitors. These are considered to be general inhibitors of dehydrogenases and to exert their action by being adsorbed on the active enzyme surfaces.

High concentrations of ethyl urethane are necessary to inhibit appreciably oxygen uptake by the rhizobia. M/3 ethyl urethane usually gives about 50 per cent inhibition of both glucose and succinate oxidation, while a 2M/3 concentration exerts an 80 to 95 per cent inhibition. The action on succinate is slightly more marked than on glucose. This is especially noticeable with succinate oxidation by *R. leguminosarum* 317. Cultured organisms of *R. leguminosarum* 317 are more inhibited than are suspensions of this species from vetch nodules. This is the only instance noted of an appreciable difference between cultured and nodular organisms with ethyl urethane.

Iodoacetate is believed to retard glycolysis by stopping phosphorylation, and it inhibits a number of dehydrogenases. Since inhibition of some dehydrogenases may be overcome by addition of reduced glutathione, the action of iodoacetate on these enzymes has been ascribed by several investigators to binding of sulfhydryl groups (Elvehjem, Wilson, 1939).

The presence of M/100 iodoacetate causes 50 to 90 per cent inhibition in oxidations by the rhizobia, the highest inhibition appearing with succinate as the substrate. The response with *R. japonicum* 505 and soybean nodule organisms is remarkably constant on both substrates. With *R. leguminosarum* 317 the cultured organisms are more sensitive on succinate than are the other organisms examined, a result similar to that observed with urethane.

TABLE III

Effect of inhibitors on respiration by "cultured" rhizobia.

Inhibitor	Final Conc.	Strain of Organism															
		100		C-1		202		209		317		333		505		602	
		G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S
Cyanide	M/100							100		98	97			100		80	97
	M/1000	99	98	98	96			94		96	96	89	94	98		70	98
	M/10000	8	27					85		76	76	−2	13	81		17	69
	M/100000			−1	59			19		5	32			21		5	30
Sodium azide	M/100	87	90	86	92	64		69		77	95	75	88	71	90	9	85
	M/200					39		62		58	94			52	82	22	67
	M/1000	57	71	37	81	−6		11			78	10	54				
Na fluoride	M/12.5	33	82	78	83					35	84	58	38	0	42	98	43
	M/25	2	39	28	40					−6	38	−3	−11	−21	12	25	5
Na iodoacetate	M/100	58	88	89	95	48		55		67	94	49	80	85	84	83	81
	M/1000	16	76	52	85	27		29		33	75	20	55	60	44	44	45
Na malonate	M/5	31	74	65	94		86			12	89	82	89	38	34		
	M/10						77			−9	76			−36	−5		
	M/25	3	57	17	82	−5	24	41	23	−12	−15	25	20	−67	−8		
Na maleate	M/12.5					−6		−14		−4	30			−154	−2		
Na pyrophosphate	M/12.5	−20	17	−35	18					22	87	36	50	12	7	−128	9
Ethyl urethane	2M/3									99	97			79	92		
	M/3	28	70	25	71			48		54	91	19	55	15	57		

G = Glucose used as substrate   S = Na succinate used as substrate

Warburg flasks contain: 1 ml. pH 6.5 M/15 phosphate buffer, 0.5 ml. M/25 glucose or 0.5 ml. M/25 neutral sodium succinate, 0.5 ml. neutral inhibitor, 1 ml. bacterial suspension, and 0.15 ml. 20 p.c. KOH in inset cup. (The 20 p.c. KOH in inset cup was replaced with KOH-KCN mixtures in measuring cyanide inhibition; Krebs (1935)). The buffer, inhibitor and bacteria were mixed before the substrate was added. Data recorded as per cent inhibition. Negative values indicate stimulation.

3. *Succinodehydrogenase inhibitors.* Malonate is considered a rather specific inhibitor of succinic dehydrogenase though it is also reported as inhibiting lactic and malic dehydrogenases (Elvehjem, Wilson, 1939). With the root nodule bacteria, malonate, far from being an inhibitor, is a good substrate in ordinary concentrations, especially for the slow-growers. Only when malonate was employed in very high concentrations, such as M/5 and M/10, could any inhibition be detected. Even at these concentrations, the cultured cowpea organisms and vetch nodule organisms showed a stimulation on glucose and to some extent on succinate. Only with cultured *R. leguminosarum* 317 and *R. trifolii* 202 on succinate is the inhibition really striking. In any case the observed inhibitions are of doubtful significance because of the high concentrations of malonate necessary. Inhibition of succinic dehydrogenase, however, may be masked by the utilization of the malonate itself.

Under anaerobic conditions, the results are quite different. Tam (1939) has found that dehydrogenation of both glucose and succinate in the presence of methylene blue by suspensions of the resting cells of *R. trifolii* 209 is inhibited 40-50 per cent by M/25 malonate. At M/100 concentration, inhibition is still evident.

Maleic acid, which is also regarded as an inhibitor of succinic dehydrogenase, is likewise oxidized by the rhizobia. M/12.5 maleate has little effect on *R. trifolii* and *R. leguminosarum,* but is markedly stimulating for *R. japonicum* on glucose. The oxygen uptake by organisms from cowpea nodules is stimulated four-fold by this inhibitor (Thorne and Burris, 1938).

Pyrophosphate is another succinic dehydrogenase inhibitor. Except for *R. leguminosarum,* but slight inhibition of succinate oxidation occurs, even at a concentration of M/12.5, and little effect on glucose metabolism is observed. Moreover, a two-fold increase in O_2 uptake by the cowpea organisms is induced. Stimulation of glucose metabolism by pyrophosphate has been observed by others and has been interpreted as resulting from the binding of iron and copper.

Tam (1939) has found that methylene blue reduction by *R. trifolii* 209 is stimulated 40 per cent on glucose and 50 per cent on succinate in the presence of M/100 pyrophosphate.

Although studies of inhibitors do not give a clear-cut picture of the enzyme systems primarily responsible for the respiration of the rhizobia, they aid in the selection of the possible courses of the reactions. The presence of cytochrome bands in the organisms and the strong inhibition by cyanide point to the importance of the cytochrome system in the respiration. This is largely confirmed by the inhibition exerted by azide, except for the anomalous behavior of the organisms from vetch nodules.

The rapid metabolism of succinate by all the organisms studied would suggest that it plays an important part in the general respiration of the cells. Since the 4-carbon acids other than succinate are also excellent substrates, it might be supposed that the 4-carbon dicarboxylic acid cycle is important in the respiration of the rhizobia. Though the studies of substrates indicate such possibilities, they are not confirmed by the action of the inhibitors believed to be specific for succinic dehydrogenase. Malonate, maleate and pyrophosphate are only weakly inhibitory or are even stimulatory. However, this circumstance does not entirely rule out the 4-carbon acid cycle, for the enzymes in the intact cell, as was used in these studies, may be very resistant to inhibitory action, or an alternative mechanism may be partially replacing the system inhibited.

Discussion

As was indicated above, these studies were designed to answer three specific questions, and attention may now be directed toward the answers which the available data afford.

The first question: "Is the respiratory system of the root nodule bacteria similar to that found in other microorganisms and tissues, or is the function of nitrogen fixation uniquely associated with an unusual type of respiration?" These studies show that the respiratory system of the rhizobia is very flexible in that it can metabolize a great variety of substrates, but the same may be said of numerous soil microorganisms. Responses of the respiratory systems of the rhizobia to changes in the physical environment have not been unusual. Aside from the stimulation of the organisms from vetch and pea nodules by azide, and the rapid oxidation of malonate and maleate by the slow-growers, there appear to be no particularly unique features in the respiratory mechanism of these bacteria. It is doubtful whether the few peculiar responses observed have a significance for the nitrogen fixation reaction.

The second question: "Are there fundamental variations in the respiratory systems of the species and strains of the root nodule bacteria which might be correlated with specific differences in their ability to fix nitrogen in association with the various legumes?"

Survey of the results of the studies on oxygen uptake by good and poor strains of rhizobia from several cross-inoculation groups indicates a definite trend in the responses as we pass from the fast-growers to the slow-growers. The typical fast growers, clover and pea organisms, have the lowest pH optima; that of the intermediate alfalfa

organism is somewhat higher, and the typical slow-grower, the soybean organism, shows maximum respiration at a distinctly higher pH. Clover, pea and alfalfa respond in a very similar manner to changes in the oxygen tension, but alfalfa 101 respires well at lower oxygen tensions and thus resembles the slow-growers. The qO_2 values on glucose show a progressive drop from the typical fast-growers through alfalfa to the typical slow-growers. The inverse trend is observed in the ability of the different species to oxidize organic acids.

The relatively low rate of oxidation of glucose and the correspondingly better utilization of the organic acids by the slow growing organisms, whether grown in the laboratory or in the nodules of their host plant, suggest a difference in the carbon metabolism of the plants which serve as hosts to the slow growers as compared with those for the fast-growers. Such a mutual adaptation may be of importance in governing the initial infection of the host plant and subsequent effective nitrogen fixation.

With the possible exception of the response to changes in the pO_2 by *R. meliloti* 100 and 101, no significant difference between good and poor strains of rhizobia within a species has been evident. The data strongly support the view that few, if any, differences exist in the metabolism of these two types of bacteria.

To the third query: "Do differences exist in the respiratory systems of a given strain when grown on laboratory medium in comparison with growth in association with the plant?", a quite definite negative answer is indicated. Aside from differences in adaptation to oxygen tension, the responses of the cultured and nodular organisms to all the factors studied are remarkably similar. Added confidence in the use of previous observations concerned with the physiology of the bacteria which were obtained with laboratory cultures is thus provided by this demonstration. Nevertheless, it is believed desirable, whenever possible, to study the activities of the organisms which have grown under natural conditions in the plant rather than in the somewhat artificial environment of the laboratory.

Projected Studies

Following this survey which compared the properties of the various types of the root nodule bacteria, attention can now be directed toward studies which should have a more direct bearing on the questions concerned with the mechanism of symbiotic nitrogen fixation. In order to obtain significant information on these questions it is believed that detailed examination of the metabolism of a few representative species should be undertaken. In such studies *intensive* investigation of

the manner in which the organism obtains its energy and transforms nitrogenous compounds will be emphasized rather than *extensive* studies on the general properties of the respiratory system. As examples of this approach to the problem of the mechanism, some preliminary data on two projects which are now under investigation will be briefly cited.

Anaerobic glycolysis.

The root nodule organisms are classed as strict aerobes, but it has been reported that heavy suspensions will attack glucose under anaerobic conditions. This observation would have little significance except for the fact that the nodule on the host plant may be regarded as essentially a mass of resting cells. It is of interest, then, to determine if suspensions of the organism prepared directly from the nodule will attack glucose anaerobically. If suspensions of either cultured or nodular organisms are placed on glucose in an atmosphere of nitrogen, CO_2 is liberated, indicative of the formation of acid. Quantitatively, the apparent glycolytic activity is higher in the cultures kept on laboratory medium, but the faculty is possessed by all suspensions of the root nodule bacteria tested, irrespective of whether they were grown on artificial medium or in the host plant. These anaerobic qCO_2 values are from 1/2 to 1/6 as great as the qO_2 values of the same bacteria (Wilson, 1939).

Since glycolysis releases a small amount of energy compared to that obtained by complete oxidative metabolism and proceeds at a much slower rate than does oxygen uptake, and since the presence of oxygen would likely inhibit glycolysis through the Pasteur effect, ordinarily the quantitative significance of glycolytic activity in the nodule probably would be small. Nevertheless, the ability of the organism to attain its maximum rate of aerobic respiration at a very low pO_2 and its possession of an anaerobic respiration mechanism may be important for its function in the nodule as the pO_2 in the interior of this tissue would probably approach zero.

Respiration stimulants

Allison, Hoover and Burk (1933) reported the occurrence of a compound which they designated as *Coenzyme R* and which they stated acted as a growth factor for the root nodule bacteria by virtue of its stimulation of respiration. Since West and Wilson (1939) found that the activity of Coenzyme R for growth stimulation can be replaced with biotin concentrates, a study of the effect of such concentrates on the respiration of glucose by suspensions of *R. trifolii* was undertaken.

When an extremely active biotin concentrate

from egg yolk was used in quantities 1 to 100 times that required for half-maximum stimulation of growth (0.01 to 1 microgram per flask), no effect on respiration was observed. Likewise, preparations of Coenzyme R were without action on respiration of the resting cells when used in quantities comparable to that required for growth stimulation. These results were not unexpected since the resting cells had been grown in the presence of a high concentration of yeast extract and accordingly should not lack the accessory growth factors. However, less pure fractions of biotin, in which low concentration of the growth factor made it necessary to add 50 to 100 micrograms per flask, caused a marked stimulation in the rate of respiration. No correlation was found between the activity of a fraction in stimulation of growth and of respiration.

With the most active fraction for the stimulation of respiration (biotin fraction VIII) additions of 3 to 166 micrograms per flask caused a linear increase in the uptake of oxygen by *R. trifolii* 205 ranging from 15 to 120 per cent (glucose substrate).

Attempts to replace this crude fraction with the following known growth stimulants were unsuccessful: sporogenes vitamin, β-alanine, pantothenic acid, thiamin, riboflavin, cozymase, cocarboxylase, nicotinic acid and inositol. All were inactive even in concentrations much higher than those used for maximum growth stimulation. The greatest stimulation, obtained with 100 micrograms of inositol, was only 12 per cent.

As the response to known growth factors was extremely slight, it was thought possible that the stimulation arose from a carbohydrate or an organic acid acting as a substrate. It will be remembered that succinate and a few other substrates are superior to glucose for the respiration of *R. trifolii*. A number of substrates including succinate, fumarate, malate, pyruvate, arabinose, lactate and aspartic acid were tested by adding 100 micrograms of each per flask. Succinate and arabinose gave about 20 per cent stimulation, aspartic acid, somewhat more, and the others, little or none. As the activity of both succinate and aspartic acid was considerably less than that of the biotin fraction, a number of other compounds, mostly amino acids, were tested in an effort to discover a more active substance. The results showed that asparagine, histidine and arginine are very stimulatory and that casein hydrolysate would completely replace the stimulating activity of fraction VIII.

In order to ascertain how rapidly the response occurred, a very heavy suspension of *R. trifolii* was prepared. Readings on the respirometer were made at four minute intervals. As is evident from the data in Fig. 4, up to 16 minutes the
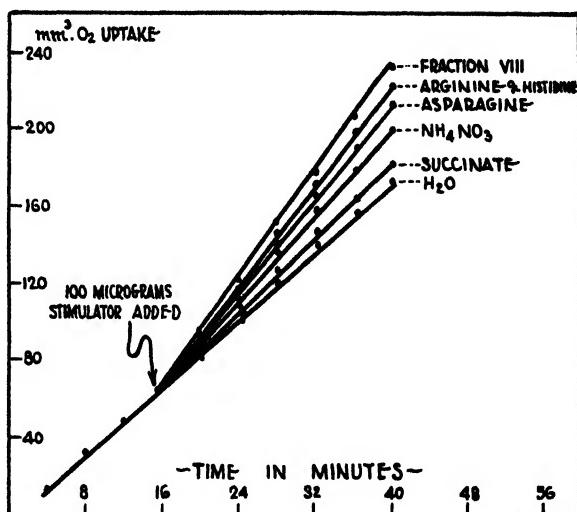
Fig. 4. Effect of addition of various substances on respiration of *R. trifolii*.

curves were identical; at this point the substances to be tested were added from a side-arm, and the response was immediate. Both arginine and histidine closely approached the stimulation induced by the biotin fraction.

From these data it has been concluded:

1. The stimulation of respiration by 50 to 100 micrograms of added materials may not necessarily indicate the action of a growth substance since the effect can be duplicated through the addition of known compounds which have no apparent stimulatory effect on growth of the root nodule organisms. It should be noted that the reported activity of preparations of Coenzyme R on respiration is certainly not due to the nitrogen-containing compounds found effective in this work since most Coenzyme R preparations are essentially nitrogen-free. Although biotin concentrates which are active in growth experiments are without effect on respiration, this result may arise from the method of preparing the resting cells, *i.e.*, growing the organisms on yeast extract.

2. The action of the nitrogen-containing compounds found effective in stimulating respiration does not appear to arise from cell division because: (a) the response is immediate, whereas the usual generation time of the organism is 2 to 3 hours; (b) in the heavy suspensions used, increase in number of cells would not be expected to occur; (c) the quantity of nitrogen added is very small (about 25 micrograms) in comparison with that already present in the cells (1200 micrograms); (d) the uptake of oxygen remains linear with no indication of logarithmic growth. In general, the effect of the stimulants appears to be more comparable to the specific dynamic action of amino

acids in animal metabolism than to an effect on cell division.

Further investigations of these substances which act as respiratory stimulants for resting cells of *R. trifolii* are now under way. Of particular interest are: whether a nitrogen group is essential for the activity; whether the method of cultivating the organisms with reference to growth factors will affect the results; and whether these compounds function in the same manner when in contact with the organisms in the host plant. It is noteworthy that the compounds which were found most effective in stimulating the respiration occur in high concentration in the nodules of leguminous plants.

Summary

The methods developed for study of respiratory enzyme systems have been applied to certain problems concerned with the mechanism of symbiotic nitrogen fixation. Such application includes comparisons of the biochemistry of: (a) different strains and species of the bacteria; (b) bacteria grown in the laboratory and in association with the host plant. Other applications deal with studies on the anaerobic metabolism of the organisms and the nature of certain substances which stimulate their respiration.

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DISCUSSION

Dr. Hogness: In studying the effect of inhibitors on the succinic dehydrogenase system we tried malonate, citrate, oxalate, and some other salts of organic acids and found a rather interesting effect; with a very small addition of the inhibitor there was always an acceleration before the inhibition set in. We found some preparations which did not show as large an initial increase as others. We assumed that the effect was due to copper, and by adding copper to the latter preparations we simulated those preparations which showed this large initial acceleration. We suspected that the initial increase was due to copper because it had previously been shown that copper is a strong inhibitor for this reaction. The malonate, citrate and oxalate combine with the copper ion and destroy its inhibiting effect. An apparent acceleration would be the result of the addition of these salts. In your case you were using far larger concentrations of malonate than we used. According to Szent-Györgyi's theory we might assume that the partially completed copper complex could also act catalytically.

Dr. Burris: The results we observed were somewhat different from yours. Malonate was used in high concentrations and the stimulation persisted, whereas you noted an initial stimulation and a subsequent inhibition. In our case it would seem that the malonate stimulated oxygen uptake by acting as a substrate rather than by affecting another inhibitory substance, since only the slow-growing rhizobia were stimulated, and these bacteria oxidize malonate quite readily.

Dr. King: I think there is a much simpler and more general explanation for most of these phenomena where the inhibitor shows an initial stimulus to the reaction which at higher concentration shows an inhibition. I believe it is a phenomenon which is also recognized widely in pharmacological investigations of living organisms. It seems that many compounds which exert an inhibition of enzyme activity either in solution or in bacterial suspensions will show an acceleration at low concentrations. The same thing is true of many poisons. In a living organism you get a range of stimulation followed by a range of inhibition or toxicity. I think that the phenomenon may be due to the activating complexes of the inhibitor itself.

Dr. Hogness: If I recall correctly cyanide also accelerates.

Dr. Barron: HCN in small concentrations accelerates the respiration of rabbits; at higher concentrations it kills them.

Regarding the question of the catalytic action of the C_4 dicarboxylic acids it seems to me that if you want to see the catalytic effects of these acids, instead of using an indirect method, such as the effect of inhibitors, you ought to test their action on the rate of oxygen consumption in the presence of glucose. We might say that in quite a number of experiments on the effects of the C_4 dicarboxylic acids in bacterial respiration, we have not found a single case where the C_4 dicarboxylic acids would stimulate the rate of oxidation of glucose. In every case the rate of oxidation of glucose plus C_4 dicarboxylic acid could be accounted for entirely by an additive effect. Banga and Califano have recorded similar negative tests on the respiration of *B. coli*.

Have you done any experiments on the oxidation of amino acids and the "Umaminierung" process? What is the rate of synthesis of amino acids in the presence of pyruvic acid which occurs in slices and in tissues?

Dr. Burris: We have tested the catalytic effect of the 4-carbon dicarboxylic acids on glucose oxidation and have failed to observe an increase in oxygen uptake above that which would arise from the direct oxidation of the solids.

In our laboratory Watson and Wyss have been studying the "Umaminierung" process. We are chiefly interested in whether aspartic acid may arise by the transfer of nitrogen from amino acids to oxalacetic acid. Such a transfer has been observed in the presence of macerated nodules, but as yet we have not demonstrated the transfer by rhizobia grown in laboratory media.

Dr. Hogness: I am interested in the μ values which you obtained with the different bacteria. These values are presumably a measure of the heat of activation of the slowest process taking place in the respiratory chain. There is a considerable difference between them: from 7,000 to 10,000 or 12,000 in the one case and 21,000 in others. I wonder whether anybody knows of a heat of activation as high as 21,000 for enzymatic processes.

Dr. Burris: The μ value of 21,000 calories, to which you refer, was obtained in the temperature range 5.2°-15° C. Crozier finds that biological reactions frequently show very high μ values at low temperatures. This may well be an artifact which arises from the method of plotting the experimental values. As pointed out, Belehradek contends that μ values are not a true measure of activation energy. Plotting our data by his empirical formula we obtained a straight line for the entire temperature range studied.

Dr. Hogness: Consider a series of related reactions. At the low temperatures the slowest reaction may have the largest activation energy. As the temperature is increased the velocity of this reaction will increase at a greater rate than the others and another reaction, with smaller activation energy, may now become the rate-determining step. The results are just what might be expected from such a concept. On the basis of related chemical reactions one would not expect a sharp break but, rather, a gradual transition from one slope to another.

Dr. Ponder: In many cases, I think that a gradual transition is exactly what one gets experimentally, and that the breaking up of the curve into two or more straight lines is merely a convenient device without reality behind it. The determination of the position of the "breaks" and of the slopes of the lines is, to me, much too arbitrary; Korr, following up a suggestion made by Gerard at the 1936 Symposium here, tried to find to what extent different people would agree as to the slopes of such lines, with discouraging results (J. Cell. and Comp. Physiol., *10*, 461, 1937). The whole matter was discussed at the Symposium in 1936, and the issue seems to me to turn on the very point which Hogness has brought out, *i.e.* the existence of gradual transitions rather than sharp breaks.

Dr. King: I should like to ask if you have tried, or might be willing to try, the effect of these organisms on oxidizing the longer chain dicarboxylic acids. It seems to me that you may have a very valuable method for studying the oxidation of fatty acids, especially those with long hydrocarbon chains between two carboxyl groups. That is a field where little headway has been made, and where those who have attempted to follow the metabolism of fatty acids are in something of a quandary. Verkade has done some work, but the tools available are very limited, and very few investigators have been able to get clear-cut results.

Dr. Burris: The highest dicarboxylic acid tested was citric acid. The higher fatty acids have not been studied. One would not expect the rhizobia to be suitable organisms for the investigation of such oxidations, though some of the lipolytic bacteria might be profitably used.

Dr. Barker: Have you any concept as to what metabolic activities the nitrogen fixation is tied up with? I believe that was the starting point for the investigation. Is it dependent on respiration or will it take place anaerobically? Do you have any information as to what controls or directs the fixation process?

Dr. Burris: Apparently respiration supplies the energy for symbiotic nitrogen fixation. Symbiotic fixation does not occur anaerobically, though certain of the free-living anaerobes (*Clostridium sp.*) fix nitrogen in the absence of atmospheric oxygen.

The effect of the pressure of oxygen and nitrogen on symbiotic nitrogen fixation has been studied by supplying different levels of the gases to red clover plants grown in closed bottles. Fixation is independent of oxygen tension between 0.05 and 0.4 atmosphere; below 0.05 and above 0.4 atmosphere the rate of fixation drops sharply. You will recall that the rate of respiration of bacteria taken from nodules is as great at 0.05 atmosphere oxygen as at higher levels. Symbiotic fixation is independent of nitrogen pressure above a pN_2 of 0.1 atmosphere.

Many phases of the problem of symbiotic nitrogen fixation remain to be clarified. Theories of the mechanism of fixation have been discussed by Wilson (Ergebnisse der Enzymforschung, *8*, 13, 1939).

THE REVERSIBLE HEAT ACTIVATION OF RESPIRATION IN *NEUROSPORA*

David R. Goddard

Warburg (9) and Runnstrom (6) have shown that a large increase in respiratory rate occurs upon fertilization of sea urchin eggs, and Bodine (2) has shown that the respiration of diapause grasshopper embryos is low and that a marked increase in respiratory rate occurs when diapause is broken. A comparable case has been discovered in the ascospores of the fungus *Neurospora tetrasperma*. *Neurospora* produces two types of spores, conidia and ascorspores. Shear and Dodge (7) showed that the conidia germinate readily upon transfer to culture solutions but that the ascospores are dormant, and that these dormant spores may be induced to germinate by a short period of heat treatment. Goddard (3) and Goddard and Smith (4) have shown that the activation of the dormant spores is reversible, and that following activation there is a large increase in respiratory rate and initiation of fermentation.

The Activation of Germination

In this paper some results obtained on activation and the concomitant changes in respiration and fermentation will be presented. If the ascospores are heated in thin-walled capillary tubes for 20 minutes at various temperatures, and then transferred to hanging drops at room temperature, germination commences about 3 hours later and is complete in about 6 hours. The germination response to heating is shown in Fig. 1. Each point is based on counting 350-500 spores. The spores may be heated and planted in distilled water, inorganic buffers, or nutrient solution without any change in the shape of the curves. The extreme steepness of the curve at 50-52° C. gives

the appearance of a critical temperature. Shortening the time of heating displaces the curve to the right without change in shape. Lengthening the time of heating displaces the curve to lower temperatures but maximum activation cannot be obtained below 47° C. regardless of the time of heating.

Similar experiments were conducted in which the time course of activation was determined at several constant temperatures. The results of one such experiment are shown in Fig. 2, from which
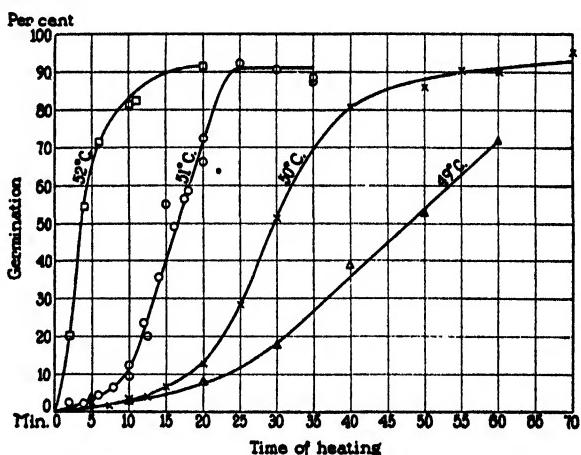
Fig. 2. The effect of time of activation at constant temperature on the percentage germination. Same spore lot used for all curves. (J. Gen. Physiol. *19*, 48, 1935.)

it may be seen that the curves are S-shaped. The temperature range over which such experiments may be conducted is limited to approximately 47-52° C. At higher temperatures activation is too fast to time readily; at lower temperatures the process is extremely slow and the activation is never maximal. The times of heating to induce 50 per cent germination are compared for t_1 and $t_1 + \Delta t$, thus an apparent temperature coefficient (Q_1) is obtained (Table I). It will be observed that the apparent temperature coefficient is not constant; this will be discussed below.

The time between activation and germination is independent of the percentage of the spores activated; that is, judged by germination, either a spore responded to activation completely, or not at all. After partial activation the ungerminated spores could be induced to germinate by a second heat treatment of longer duration or of higher temperature. To date no chemical or physical means other than heat treatment has been found to induce germination.

The activation occurs as readily with the spores
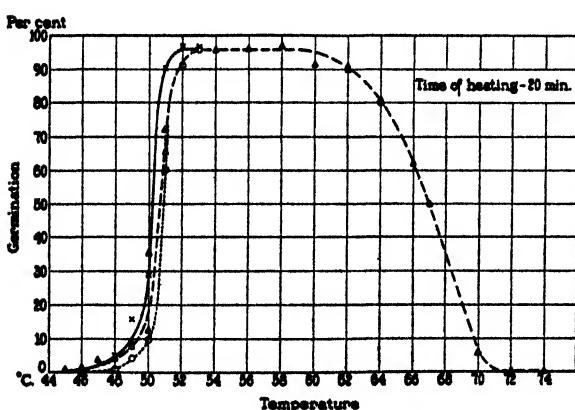
Fig. 1. The effect of the temperature of activation on the germination. Germination at room temperature, counts made 6-8 hours after activation. Three separate spore lots. Each point is based on counts of 350-500 spores. (J. Gen. Physiol. *19*, 48, 1935.)

TABLE I

Temperature of activation	Time of activation to induce 50 p.c. germination	"Q₁"
° C	Mins.	
45	2820	2.77
49	48	1.65
50	29	1.70
51	17	4.85
52	3.5	

"Q_1" = apparent temperature coefficient per 1° C;
$Q_{10} = (Q_1)^{10}$.

in pure nitrogen as in air, or in the presence of cyanide. However, if the activated spores are maintained for an appreciable time after activation under anaerobic conditions or in cyanide, they de-activate. Typical experiments are shown in Table II and Fig. 3. After the designated times in nitrogen or cyanide the spores are placed in air, or freed of cyanide; and 6 to 8 hours after returning to air the germination counts are made. That this is de-activation and not death is shown since with a second heat treatment these spores germinate normally (Table II). Thus the activation process itself is not oxygen dependent, but the process following activation and leading to germination depends upon oxygen and is cyanide sensitive, and the inhibition of this process results

TABLE II

Reversible de-activation of germination

	Time in N after activation	Germination	Re-activated germination
Nitrogen series	hr.	p.c.	p.c.
Control (activated in air)	—	94.2	
Activated in N	0.0	95.9	
	0.5	96.3	
	1.0	96.7	
	2.0	74.9	
	3.0	41.1	
	4.0	2.7	
	9.0	1.6	92.5
	50.0	0.0	94.7*
	24.0	4.1	91.5**
Cyanide series (HCN = 1 × 10³M)	Time in HCN after activation		
Control (activated in air)	—	96.6	
Activated in HCN	0.0	95.6	
	0.5	93.7	
	1.0	85.6	
	1.5	75.5	
	3.0	52.8	94.5
	4.0	37.4	
	7.0	0.0	

* A duplicate tube was re-activated and set aside unopened for an additional 24 hours, and the data are given in the next line below.
** These spores have been activated 3 times and de-activated twice.
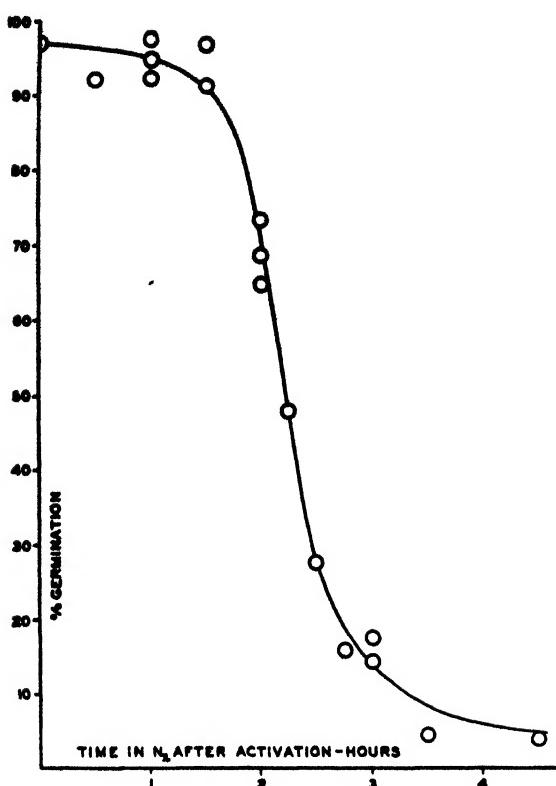
Fig. 3. Time course of de-activation in nitrogen at 25° C. Spores activated at 56° C. for 20 minutes, distributed into several vessels and made anaerobic with purified nitrogen. After the indicated time, transferred to air; germination after 6-8 hours in air.

in de-activation. The activation process itself is reversible. De-activation has a temperature coefficient (Q_{10}) of 2.0 between 15° and 25° C. and of 1.6 from 25° to 35° C., as determined from the time required to cause 50 per cent de-activation.

The Activation of Respiration

The respiratory rate of dormant spores and a sample of the same suspension after maximum ac-

tivation (56° C. for 20 minutes, see Fig. 1) was determined at 25° C. in the Fenn microrespirometer. The results of such experiments are shown in Fig. 4 and Table III. The spores were suspended in M/60 phosphate buffer at pH 5.6, without added substrate. It is seen that the dormant spores have a low but measureable oxygen consumption, while the heat treated spores have a rate 10 to 40 times greater. About 2 hours after activation the heated spores undergo a secondary increase in rate, about doubling the activated rate. This second increase corresponds approximately with the time when the first microscopically detected germination begins. The results of several experiments are shown in Table III. (No significance is to be attached to the intersection of the two linear curves in Figs. 4 and 11. They
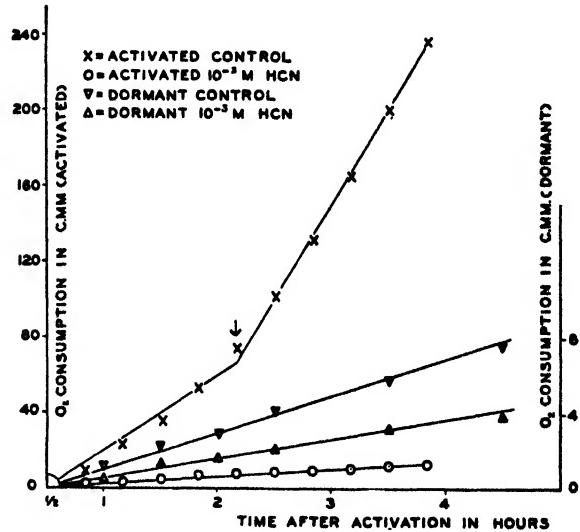
Fig. 4. O_2 consumption of activated spores (left axis), and of dormant spores (right axis). The arrow indicates the approximate time of germination. 6.48 mg. spores in each vessel. Activation for 20 minutes at 56° C.; O_2 measurements at 25° C. (Plant Physiol. *13*, 242, 1938.)

TABLE III

The effect of activation on respiratory rate

(Oxygen consumption as Q_{O_2} at 25° C.)

Spore Lot	Dormant	Activated 1-1.5 hrs. after activation	Germinating 3-5 hrs. after activation	De-activation	Re-activation
A	0.56	7.00	—	1.00	7.15
B	0.25; 0.27	10.86	19.45	—	—
C	0.53; 0.59	4.48	9.62	—	—
D	0.24; 0.27	10.36	19.60	—	—
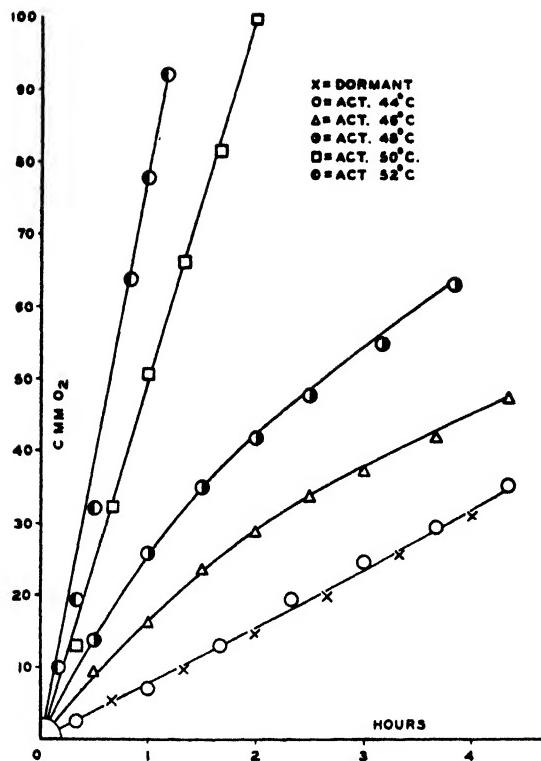
Fig. 5. Oxygen consumption of spores at 25° C. after activation for 20 minutes at the indicated temperatures. Same spore suspension used in all cases; 15.2 mg. spores.

are so drawn for graphical convenience. The arrow indicates the first visible germination.)

When samples of the same spore suspension are heated at the indicated temperatures for 20 minutes and oxygen consumption measured at 25° C. the results shown in Fig. 5 are obtained. It is observed that heating to 44° C. induces no change in the respiratory rate, while at 46° there is an initial stimulation and then a return to the dormant rate. No germination occurred at 44° or 46° C., so at the later temperature there was a temporary respiratory activation without activation of germination. Spores heat-treated at temperatures higher than 48° C. never show a decline in respiratory rate; the curves are at first linear and later increase. A series of experiments similar to those shown in Fig. 5 was run, and the rate was calculated as Q_{O_2}* on the initial approximately linear phase of the curves. Germination was determined on each vessel. Fig. 6 shows the respiratory activation and germination on the same spores. Inspection of Fig. 6 shows that respiratory activation occurs at slightly lower temperatures than does activation of germination. How-

$$* \; Q_{O_2} = \frac{mm.^3 \; O_2 \; per \; hr.}{mg. \; dry \; weight}$$

ever, the activation of the respiratory mechanism is also strongly temperature dependent.

The time course of respiratory activation at 51° C. is shown in Fig. 7. Samples were withdrawn from the heated suspension at the designated time and plunged into ice water, and after cooling transferred to the respirometer vessels. There was a time lag of approximately two minutes in bringing the temperature of the spore suspension to that of the water bath. The O_2 consumptions were plotted as in Fig. 5 and from the initial slopes of the curves the Q_{O_2} values were calculated. Several experiments yielded curves of the shape shown in Fig. 7, but the absolute values varied rather badly. However, none of the curves was S-shaped as are the germination curves, nor did any of them show an initial lag phase. The ratio of the times of heating to bring the Q_{O_2} to 4.0 (about 50 per cent of the maximum) at 50° to 51° C. is 1.8, giving an apparent Q_{10} of activation of 350. Since a slight error in determining the time of activation or the respiratory rate will introduce a large error in the Q_{10}, the above value shows only the order of magnitude. Though the Q_{10} of activation is high, the Q_{10} of the respiratory rate of either dormant or activated spores as determined from O_2 measurements at 15° C. and 25° C. is of ordinary value, about 2.5.
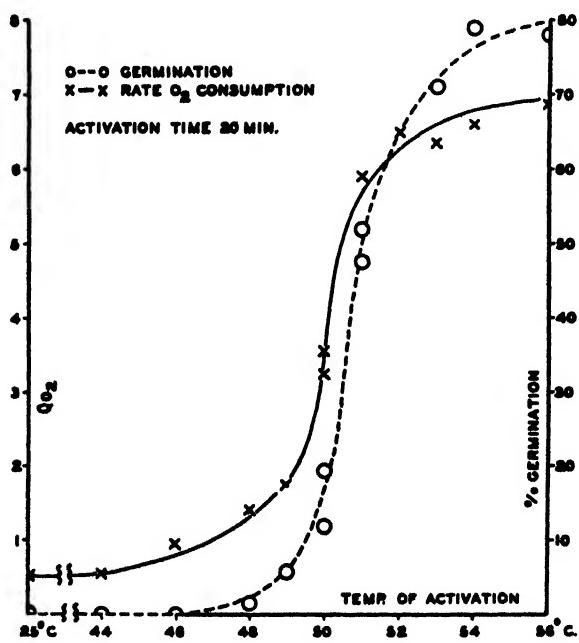

Fig. 6. Rate of oxygen consumption at 25° C. as Q_{O_2} for same spore suspension (15.2 mg.) activated for 20 minutes at the indicated temperatures. Germination counts on the same vessels as O_2 measurements. Rates calculated on the linear phase of the curves similar to those in Fig. 5.

Fig. 7. Time of activation in relation to rate of respiration at 25° C. The Q_{O_2} values were calculated from the aproximately linear phase of the respiration (15-45 minutes after activation).

The reversible nature of de-activation on the respiratory rate is shown in Table III. The activated spores were de-activated for four hours in pure nitrogen, made aerobic and the rate determined. They were then re-activated and the rate again determined.

The experiments on heat activation of the respiratory rate show that the respiratory activation is strongly temperature dependent, that the respiratory activation is reversible, that there is no lag in the respiratory activation, and that it is possible to bring about a small respiratory stimulation without activation of germination.

The Activation of Fermentation

When dormant spores are made anaerobic with nitrogen passed over heated copper, it is found that they produce no measureable CO_2. The limit of measurement is about 0.6 mm.³/hour, and for the dormant spores $Q^N_{CO_2} < 0.03$. It is found that activated spores liberated CO_2 rapidly; immediately after activation at an approximately constant rate ($Q^N_{CO_2} = 5$ to 8) and gradually the rate declines to zero. The results obtained with individual vessels, in which the spores were heated for 20 minutes at the indicated temperatures are shown in Fig. 8. Since germination will not occur in the absence of oxygen, samples were removed
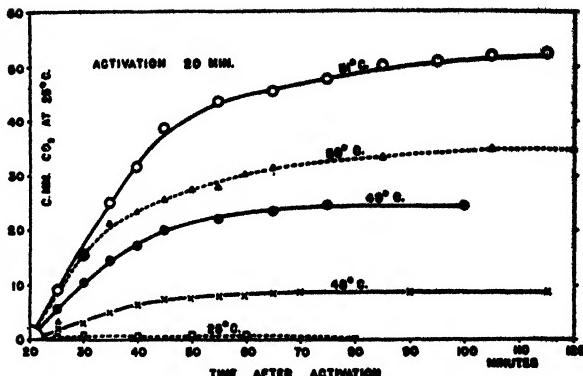
Fig. 8. Anaerobic CO_2 production of spores at 25° C. after activation for 20 minutes at the indicated temperatures. Same spore suspension in all vessels; 13.75 mg.

immediately after activation and placed in hanging drops to determine the percentage germination. The rates of CO_2 evolution, as calculated from the initial linear phase of curves like those in Fig. 8, are shown in Fig. 9. It is observed that the curve
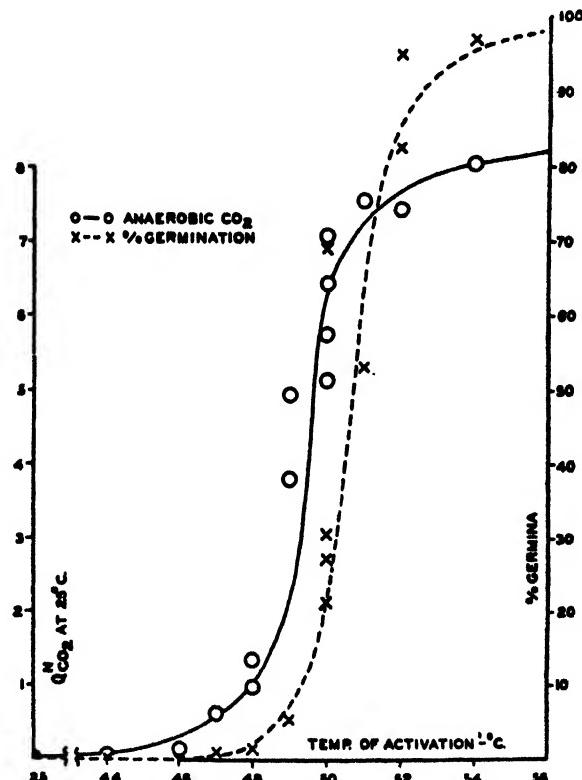
Fig. 9. Rate of anaerobic CO_2 production as $Q^N_{CO_2}$, activated for 20 minutes at the indicated temperatures. Rates calculated from the approximately linear phase of the curves as in Fig. 8. Germination counts made by removal of a sample of each suspension and transferring to a hanging drop in air.

of activation of fermentation in Fig. 9 is practically identical with the curve of activation of respiration shown in Fig. 6.

The spontaneous decline in CO_2 production is strikingly similar to the de-activation of germination. If the rate of CO_2 production is plotted against time after activation, a curve essentially like the de-activation curve of germination shown in Fig. 3 is obtained. The reversible nature of the fermentation activation is shown in Fig. 10.
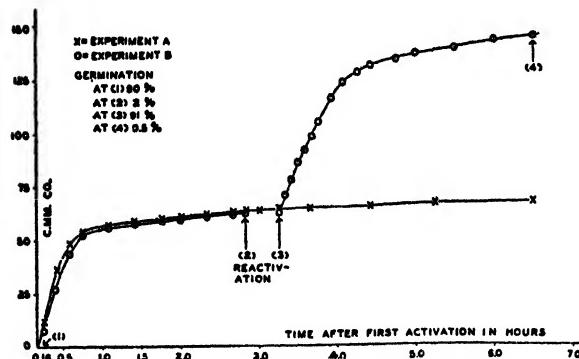

Fig. 10. Reversible activation of anaerobic CO_2 production. In experiment A, the reading was not interrupted. In experiment B, the vessels were removed at arrow 2, re-heated for 20 minutes, allowed 10 minutes for temperature equilibrium, and readings commenced at arrow 3. Germination tests were made by removing the spores to air and counting several hours later, at each point indicated by an arrow. CO_2 production measured at 25° C.; activation for 20 minutes at 55° C. (Plant Physiol. *13*, 259, 1938.)

The CO_2 evolution of activated spores was followed in 4 vessels for 170 minutes. Two vessels were then removed, germination samples taken, the spores reheated at 55° C. for 20 minutes, and the CO_2 production again followed. During this period readings were continued on the other two vessels which had not been reheated. It is seen from Fig. 10 that the reversible activation of fermentation corresponds with the reversible activation of germination. When anaerobic CO_2 production is measured at 15° C., the absolute rate is of course lower, and the rate of de-activation of fermentation is slower, with a Q_{10} of de-activation of approximately 2.0.

The de-activation of germination and respiration occurs only anaerobically, or aerobically in the presence of respiratory poisons. However, the de-activation of fermentation occurs aerobically as readily as anaerobically, as may be seen from Table IV. The activated spores were distributed in 4 vessels, one was made anaerobic and the CO_2 production followed, the others were shaken with air under conditions that give the maximum respiratory rate. Then, at 1, 2, and 4 hours after completion of activation one vessel was made anaerobic and the CO_2 production followed.

TABLE IV

Aerobic and anaerobic de-activation of carboxylase.

(Anaerobic CO_2 in $mm.^3/hr.$ at 15° C.)*

Time after activation (Hours)	Hours in air after activation			
	0	1	2	4
0.25-1.25	129	—	—	—
1.25-2.25	101	96	—	—
2.25-3.25	59	53	51	—
4.25-5.25	14	15	17	25
Germination 12 hours after returning to air*	0.37	0.10	0.10	26.0

* 26.4 mg. activated spores in M/40 pyruvate.
** Germination (in air) of control, 95.8 p.c.

Goddard and Smith (4) had assumed that because of the close correlation between the activation of fermentation and respiration and similar values of Q_{O_2} and $Q^N_{CO_2}$, that there must be a common pathway for fermentation and respiration in *Neurospora*. This need not be the case, however, for in spite of the parallelism in activation, the fermentation process may de-activate without de-activation of respiration. In fact, germinated spores which have a high respiratory rate have a very low fermentation.

The Respiratory Block of Dormant Spores

We may ask what constitutes the respiratory block in dormant *Neurospora* spores, or, conversely, what part of the respiratory mechanism undergoes heat activation? We may assume concerning the respiratory systems of dormant and activated spores, either (a) that the dormant and activated respiratory mechanisms are qualitatively dissimilar or even independent, and that heat treatment causes a *de novo* appearance of a system inactive in dormant spores; or (b) that the same mechanism is functional in dormant and activated spores, but that in dormant spores the rate is limited by some step in the total reaction chain, and that heat activation greatly accelerates the rate-determining step allowing the whole chain to proceed at the activated rate. We may ask the further question, what is the relationship between the activation process and change in respiratory rate? Does the rate depend upon the concentration of some product formed in activation? Or is the activation process a stimulatory one, and does the high respiratory rate, once initiated, maintain itself, independently of the subsequent concentra-

tion of the stimulating substance formed in activation?

Cell Surface

It is possible that the cell surface of dormant spores is relatively impermeable to O_2, CO_2 or H_2O, and that the permeability is limiting the rate of metabolism. If permeability to oxygen were limiting the rate, we should expect an increase in the partial pressure of O_2 to cause an increase in respiratory rate. The data in Table V show that

TABLE V

Effect of O_2 and CO_2 pressures on dormant spores
(Oxygen consumption as Q_{O_2})

Gas pressure	Oxygen*			CO_2†	
	A	B	C	D	E
0	—	—	—	0.33	0.71
37	0.15	—	—	0.30	0.65
75	0.15	—	0.66	0.37	0.66
150	0.17	0.27	0.65	0.30	—
190	—	—	—	—	0.58
300	0.16	0.26	—	—	—
750	—	—	0.77	—	—

* CO_2 pressure = zero.
† O_2 pressure = 150 mm.

raising the O_2 pressure from 37 to 300 mm. had practically no effect on the rate. If retention of CO_2 by the spore were limiting, it would be expected that increased external CO_2 tensions would decrease the respiratory rate. It may be seen from Table V that this is not the case. Dormant spores liberate no anaerobic CO_2, and activated spores do. The activation of fermentation cannot be explained as solely due to a change in permeability to CO_2, for the dormant spores liberate CO_2 aerobically (R.Q. = 0.85).

The experiments reported in Table VII show that ethyl alcohol and acetaldehyde produce marked increases in the rate of O_2 consumption by dormant spores. Such increases could not be obtained if the cell surfaces were limiting the rate of O_2 consumption.

Further evidence on the cell surface was obtained by determining the rate of release of oxygen from H_2O_2 by dormant and activated spores. After equilibration in the respirometer 3 per cent H_2O_2 was tipped on the spores. In 14 minutes the dormant spores liberated 126 mm.[3] of O_2; the activated spores liberated 136 mm.[3]; while an equal number of spores boiled for 10 minutes liberated 0.9 mm.[3] Unless we make the

improbable assumption that catalase is on the surface, this experiment would indicate that dormant spores are readily permeable to H_2O_2. It seems highly improbable that the cell surface is limiting the respiratory rate.

Cytochrome—Cytochrome Oxidase

If the concentration or activity of cytochrome oxidase is limiting the respiration of dormant spores, the dormant respiration should be at least as sensitive to cyanide and carbon monoxide as is the respiration of activated spores. The data in Figs. 4 and 11 and Table VIII show that this is not the case. However, if the concentration of the cytochrome oxidase is as high in dormant spores as in activated spores, only 1/8 to 1/40 of the enzyme is essential to maintain the observed dormant rate, and poisoning a large percentage of the enzyme with carbon monoxide or cyanide will have little or no effect on the dormant rate, while
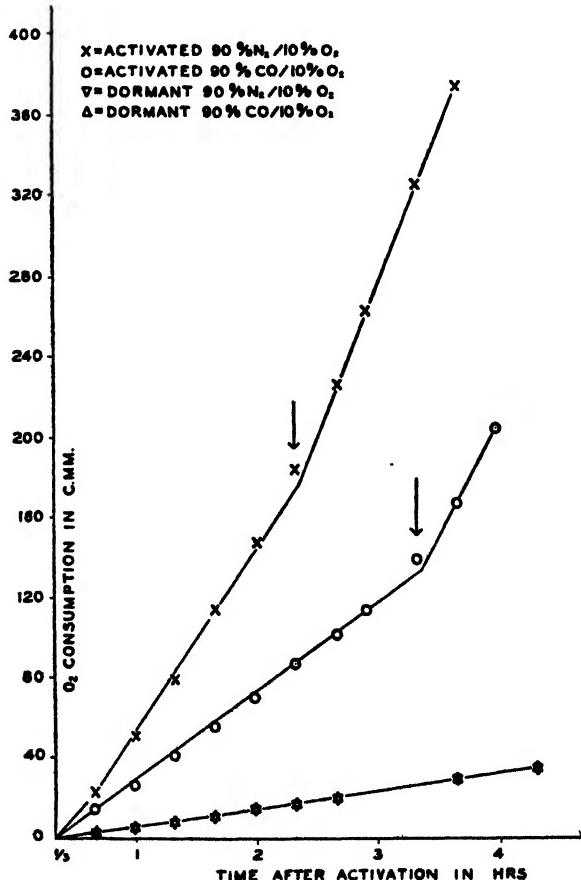

Fig. 11. The effect of carbon monoxide on the respiration of dormant and germinating ascospores, 7.95 mg. of spores per vessel in the activated experiment and 14.90 mg. of spores per vessel in the dormant experiment. The arrows indicate the beginning of germination. (Plant Physiol. 13, 248, 1938.)

a large inhibition of the activated respiration may be expected. If *Neurospora* behaves as does yeast, 10^{-4} M HCN should combine with about 50 per cent of the oxidase, and this concentration has no effect on the dormant rate. At 10^{-3} M HCN 95 per cent or more of the enzyme should be tied up, but the dormant respiration is only inhibited 47 per cent. I have interpreted the cyanide and CO experiments to mean that the respiratory rate of dormant spores is not limited by the activity of cytochrome oxidase.

TABLE VI

Effect of dyes on dormant spores
(Oxygen consumption as Q_{O_2})

Dye	Control	Experimental
Methylene blue 0.01 p.c.	0.23	0.31
Pycocyanine M/5000	0.31	0.24
Pyocyanine M/10,000	0.31	0.31
p-phenylene diamine M/1000	0.21	0.28*
p-phenylene diamine 10 mg. per vessel	0.37	0.46†

* Not corrected for autoxidation of the dye.
† Corrected for autoxidation of the dye.

The black spore walls have made it impossible to establish the presence of cytochrome, though presumably it is present. If the cytochrome activity were rate-limiting, it might be possible to increase the respiratory rate by the addition of dyes, such as methylene blue, or pyocyanine, which could be reduced by the flavin enzyme-dehydrogenase-substrate system and reoxidized by oxygen. The results in Table VI show that no appreciable change in rate occurred with added dyes. It is probable that the spores are impermeable to the added dyes, and the experiment fails to answer the question.

Dehydrogenase-Substrate Systems

The usual method of determining dehydrogenase activity is the methylene blue technic of Thunberg (8). This method was tried with dormant spores without results, for the spores removed all the dye from solution and the black wall pigment made it impossible to determine whether the dye had been reduced. No experiments were conducted with ground spores, since although such experiments might give us information on which dehydrogenase systems are present, they would not give evidence whether a particular system were active or inactive in an intact living cell. Comparable problems are found in sea urchin eggs, where the unfertilized eggs have a low respiratory rate, the fertilized ones a high rate, but the rate

TABLE VII

Effects of substrates on oxygen consumption of dormant spores
(Values given as Q_{O_2})

Substrate	Concentration	Substrate respiration	Control	Percentage stimulation
Glucose	M/50	0.345	0.355	—2.8
Sodium hexosediphosphate	M/50	0.344	0.355	—3.2
Acetaldehyde	M/50	0.707	0.355	99.2
Above three together	M/50	0.737	0.355	107.6
Ethyl alcohol	M/20	1.150	0.529	117.5
Acetaldehyde	M/50	1.152	0.529	117.8*
Alcohol plus acetaldehyde	M/20 M/50	1.178	0.529	122.8
Methyl glyoxal	M/50	0.420	0.357	15.0
Sodium succinate	M/20	0.550	0.528	4.16
Pyruvic acid	M/20	0.296	0.273	8.40
Sodium glycerophosphate	M/20	0.286	0.273	4.78
Sodium acetate	M/40	0.301	0.303	—0.6

* Stimulations as high as 228 p.c. have been obtained at M/20, and 297 p.c. at M/50 acetaldehyde.

of cytolyzed unfertilized eggs is the same as that of cytolyzed fertilized eggs (6). No information is available concerning the co-dehydrogenases or the flavin enzymes of *Neurospora* spores.

Since spores will respond to activation and germinate in distilled water, it is clear that the amount of stored food is not limiting the respiratory rate of dormant spores. However, it is possible that the rate at which the food reserves undergo phosphorylation, hydrolysis, or other anaerobic reactions leading to the final respiratory substrate, may be limiting the rate of respiration. If this were the case, the addition of the proper intermediate substrate should cause a marked increase in the respiratory rate of dormant spores. It is seen from Table VII that the only substrates which have any appreciable effect are acetaldehyde and ethyl alcohol. The increase caused by these compounds is not additive. Though the increase caused by these compounds is marked, it is far short of the stimulation induced by heat treatment. The impermeability of the spores may prevent the action of certain substrates. These results have not ruled out the possibility that the respiratory block is located in the anaerobic reactions which act on glycogen (or other food reserves) and form the actual respiratory substrate.

Anaerobic CO_2 and Carboxylase

The close correlation between activation of respiration and fermentation seems to need some explanation. Further, NaF, a powerful poison of alcoholic fermentation, poisons the respiration of *Neurospora*, as may be seen from Table VIII. Lohmann and Meyerhof (5) have shown that NaF poisons the conversion of phosphoglyceric acid to pyruvic acid, but not the decarboxylation of pyruvic acid to CO_2 and acetaldehyde.

Experiments were run to determine the effect of NaF on the anaerobic CO_2 evolution of activated spores and a high inhibition was obtained as is shown in Fig. 12. If the anaerobic CO_2 is formed by carboxylase catalysis of the reaction:

$$CH_3COCOOH \rightarrow CH_3CHO + CO_2$$
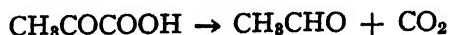
the addition of pyruvic acid to NaF poisoned spores should restore to the original value, the rate of CO_2 liberation. The curve in Fig. 12 shows that this is the case. These experiments show without ambiguity that an enzyme exists in activated spores that anaerobically liberates CO_2 from added pyruvic acid in the presence of NaF; and, therefore, conforms to the definition of carboxylase.

TABLE VIII

Comparison of dormant, activated and germinating spores

	Dormant	Activated	Germinating	Notes
Respiratory rate Q_{O_2}	0.25-0.55	4.5-10.9	9.0-22.0	
Anaerobic CO_2 as Q_{CO_2}	<0.03	5.0-10.9	1.0- 2.0	
Catalase activated as $Q_{H_2O_2}$	44.2	47.6		
Percentage respiratory inhibition by :				
0.05 M iodoacetamide	40-65	7-9	67-93	No germination
1×10^{-4} M HCN	1.7	32	55	Germination delayed*
1×10^{-3} M HCN	47.	87	94	No germination
1×10^{-2} M HCN	36.	88	96	No germination
90 p.c. CO/10 p.c. N*	4.	38	65	Germination delayed
95 p.c. CO/5 p.c. N	8.	79	82	No germination
0.1 M NaF	82	65	95	No germination
Respiratory stimulation by 0.2 M acetaldehyde	125-297	5	16	

* Delayed germination, p.c. germination = control germination but full germination occurs several hours later than in controls.
** All experiments at 25° C. except CO which were at 15° C.
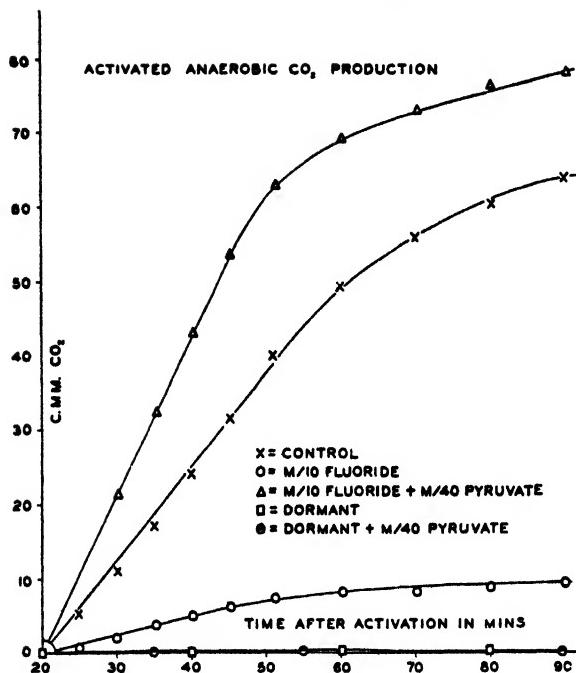
Fig. 12. Pyruvate reversal of NaF poisoning of anaerobic CO_2 production. Failure of pyruvate to induce CO_2 production in dormant spores. Activation 20 minutes at 56° C.; CO_2 measurements at 25° C. 19.5 mg. of spores in each vessel. (Plant Physiol. *13*, 256, 1938.)

If the block that prevents fermentation in dormant spores is in the processes forming pyruvic acid, the addition of pyruvate to dormant spores should induce anaerobic CO_2 production. The lower curve in Fig. 12 shows that dormant spores liberate no measureable CO_2 from added pyruvate. If we could obtain unequivocal evidence that pyruvate penetrated dormant spores at a measureable rate[1], we could say definitely that the carboxylase system is inactive in dormant spores. Since we have shown above that carboxylase is active in heat treated spores, we could also say with equal definiteness that on heat activation the carboxylase system becomes activated; and that in de-activation the carboxylase system becomes de-activated. This would be an extremely interesting case of the reversible activation of an enzyme system, within an intact living cell, and one subject to experimental control.

I do not believe that the dormant spores are impermeable to pyruvate, for we can show by the respirometer experiments that they are permeable to acetaldehyde, alcohol, ethyl urethane, CO_2, O_2, H_2O_2, and iodoacetamide. However, convincing evidence that pyruvate penetrates the dormant

[1] The Addendum at the end of this paper shows that pyruvate penetrates dormant spores. Thus we have definite evidence of the activation of the carboxylase system.

spores is not yet available. Therefore, I have provisionally accepted the view that the enzyme system, carboxylase, undergoes reversible activation in heat treatment.

The experiments do not show whether it is the protein component of the enzyme, or the coenzyme (cocarboxylase) which undergoes reversible activation. If it is the protein, there is no clear reason why activation of respiration and fermentation should be so closely correlated. There is no convincing evidence of the respiratory function of carboxylase. If it is the cocarboxylase which is responding to heat treatment, the parallelism between the activation of respiration and fermentation may be due not to a common pathway but to a common constituent (cocarboxylase) functional in both systems, for this coenzyme is active not only in fermentation but also in the aerobic oxidation of pyruvate (1).

The experiments indicate that activation of respiration, germination, and fermentation is so similar as to seem to be different responses to one fundamental process. Under anaerobic conditions we find equally good agreement in de-activation. However, the fermentation de-activates aerobically, while the respiratory rate is maintained at constant or increasing rate. Therefore, we may postulate that there is a fundamental process underlying all these effects, and we may formulate it as the conversion of some substance S to some product P:

$$S \rightleftharpoons P$$

The reaction from left to right has a high temperature coefficient, and at 50° C. the equilibrium lies far to the right. At room temperatures P is slowly converted back to S and the equilibrium lies far to the left. The reaction in both directions appears to be independent of molecular oxygen and is not poisoned by HCN.

The rate of fermentation may depend directly upon the concentration of P, or be separated from P by only a few steps. It is possible that P is the enzyme carboxylase or its coenzyme. With substrate saturation the instantaneous rate of anaerobic CO_2 production may be a measure of the concentration of P.

The initiation of the high respiratory rate following activation appears to be directly dependent upon the concentration of P, but the maintenance of respiration, once stimulated, does not depend upon the continued presence of the stimulating agent (P). (That is, the high respiration seems to be initated by the formation of P, and once initiated maintains itself.) However, if the respiration is inhibited for a considerable period, the respiratory rate remains low after removal of the inhibiting agent, and a new stimulation is necessary for return to the high rate. We may then write the scheme as follows:

$$S \rightleftharpoons P \overset{\text{aerobic}}{\rightarrow} \rightarrow \rightarrow \rightarrow \underset{\text{respiration}}{\overset{\text{activated}}{\rightarrow}} \rightarrow \rightarrow \rightarrow \text{germination.}$$
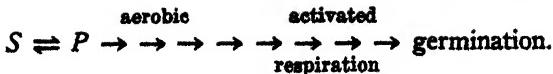
We are brought to this conclusion because activation of fermentation and respiration occur simultaneously, but fermentation de-activates reversibly in air, while the respiratory rate remains constant. Is the respiration stimulated by activation qualitatively or quantitatively different from the dormant respiration? Reference to Table VIII, which summarizes the respiratory data, indicates not only a large quantitative change, but also a qualitative change. This is particularly clear from the results obtained with iodoacetamide. We are unable to point out to what the qualitative change is due, except that it may be correlated with the ability of the activated spores to ferment.

That germination is not directly related to the concentration of P is apparent from the fact that a small activation of fermentation or respiration may occur without activation of germination. The S-shaped germination curves and the variation of the apparent temperature coefficients of the activation of germination may also be explained, if it is assumed that several steps intervene between P and germination.

A spore either germinates or remains dormant after partial activation, and the ungerminated spores will germinate after another heat treatment at higher temperature or for a longer time. Therefore, we may assume a threshold for one or more stages between heat treatment and germination. A spore in which $(P) \geqq (P_t)$ will germinate, if $(P) < (P_t)$ the spore will remain dormant [where (P_t) is the threshold concentration for germination].

The percentage of spores responding to heat treatment at constant temperature per unit time of heating fall on a normal distribution curve. Therefore, the frequencies of the concentrations of some factor X, intermediate between S and germination, form a normal distribution curve; or, among other possibilities, the value of (P_t) varies from spore to spore, along a normal distribution curve.

When spores are heated for a short time the concentration of P increases, but only in a few spores will $(P) \geqq (P_t)$; and the percentage germination will be low. During the initial stages of heating (P) will increase more rapidly than will the germination. With longer heating we will reach the midpoint of the normal distribution curve so that (P_a) will approach (P_t) [where (P_a) is the average concentration of P in the population] and a slight increase of (P_a) will mean that in many spores $(P) \geqq (P_t)$. Near the midpoint of the curve the percentage germination will increase more rapidly than (P). At the upper end of the curve we will have a symmetrical duplication of the early part of the curve with (P) increasing more rapidly than percentage germination. The threshold requirement means that very slight changes in (P_a) will mean very great changes in germination; and on this basis we may account for the extraordinary temperature coefficients of the activation of germination. The true temperature coefficient of the basic activation process $S \rightleftharpoons P$ may be high but is probably of a reasonable value. We have some evidence that the Q_{10} of this process from left to right is of the order of 1000 ($Q_1 = 2.0$) and from right to left about 2.0. If the temperature coefficient of the activation of fermentation can be determined with precision we will probably have the temperature coefficient of the activation process itself.

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Addendum by David R. Goddard, Eric Ponder, and Ephraim Shorr

The interpretation of the pyruvate experiments in the paper above is in doubt because of the absence of definite knowledge that pyruvate penetrates dormant spores. This note will show that pyruvate disappears from the supernatant solution of a suspension of dormant ascospores of *Neurospora tetrasperma*.

To approximately 3.0 ml. of well washed and centrifuged spores we added 3.0 ml. of 0.0045 M sodium pyruvate solution. The sodium salt was prepared from several times freshly vacuum distilled acid. The volume of spores in the suspension was determined by centrifuging duplicate samples at 14000 r.p.m. for 1 minute, reading, and re-centrifuging for 5 minutes more. Less than 2 per cent decrease in volume of the spores was found between the two periods. The concentration of pyruvate in the solution before addition to the

spores was determined by the bisulfite-iodine titration method of Clift and Cook[1] as modified by Elliott, Benoy and Baker[2]. The pyruvate-spore suspension was well stirred, allowed to stand one hour and the spores removed by centrifuging. The pyruvate concentration in the supernatant fluid was again determined by titration. The data in the table show that the pyruvate concentration decreased.

A similar experiment was run using the increase in electrical resistance as a measure of the decrease in pyruvate concentration. The resistance of the original solution (0.009 M) and the supernatant fluid recovered after one hour of contact with the spores was measured. For calibration the resistance of 0.009 M, 0.006 M, 0.0045 M, and 0.003 M pyruvate solutions were run. The results obtained are shown in the table.

In calculating the expected decrease in pyruvate concentration, if it were distributed equally between spore water and external solution, we have made use of the following facts or assumptions. A suspension that contains 1 ml. of spores by volume, as determined from centrifuging, has a dry weight of 0.361 gm. If we assume the density of the solids equals that of starch (1.5), then the volume of the solids = 0.24 ml. and the volume of the solvent = 0.76 ml. We have assumed that total water is solvent water.

[1] Clift, F. P. and R. P. Cook. Biochem. J. *26*, 1788 (1932).
[2] Elliott, K. A. C., and M. P. Benoy, and Z. Baker. Biochem. J. *29*, 1937 (1935).

The data obtained by titration of pyruvate and by electrical resistance both show that dormant spores are permeable to sodium pyruvate; and that the decrease in pyruvate concentration is of the order that would be expected from an unrestricted distribution between solvent and internal solution.

Discussion

Dr. Bernheim: Is it possible to do nitroprusside tests on these spores, and if so, would there be any difference between the dormant and the activated spores?

Dr. Goddard: The nitroprusside test has not been tried. I presume you are wondering whether sulfhydryl groups are formed on activation; we were unable to activate dormant spores with sulfhydryl compounds.

Dr. Shorr: Associated with your failure to get CO_2 production in the dormant spore, have you tried the effect of grinding?

Dr. Goddard: It is difficult to grind the spores unless a ball mill is available. No data are available at present on this question.

Dr. Nord: I would like to know how the experiments with pyruvate were done.

Dr. Goddard: CO_2 production was measured manometrically in a nitrogen atmosphere. The pyruvic acid was freshly distilled and neutralized. No CO_2 was liberated by dormant spores from M/40 pyruvate. With activated spores the addition of pyruvate had no effect in some experiments; in others, it stimulated by nearly 50 p.c.

A

Volume per cent of spores	Pyruvate mg per ml.			p.c. decrease in pyruvate	Calculated decrease
	Control	Experimental			
46.0	0.204	0.134			
47.8	0.205	0.147		31.3	40.2
Av. 47.9	0.2045	0.1405			

B

	Pyruvate conc.	Resistance	Relative conductance $\times 10^{-4}$		
4.06	0.009	2100	4.84		
45.2	0.006	3500	2.94		
Av. 45.6	0.0045	4300	2.46		
	0.003	6500	1.66		
	Control	2100	4.84		
	Exper.	3700	2.70	44.2	38.7

the rate of anaerobic CO_2 formation. With NaF poisoned activated spores the added pyruvate always restored the CO_2 production to the unpoisoned rate.

Dr. Nord: Henri and Fromageot (Bull. Soc. Chim. (4) *37*, 852; 1925), measuring absorption spectra, have shown that pyruvic acid *in vivo* at certain pH's is practically enolized. The biologically produced pyruvic acid contains in this dilution practically 100 p.c. enol form plus the added pyruvic acid which, as we know, is very difficult to ferment, and fermentation only occurs with an extremely high excess of yeast. It contains practically no keto form; there is, no doubt, some difference between the penetration and the permeability of the keto form and the enol form. I wonder whether it is not possible to explain the difficulties observed by you in activated and dormant spores with the different rates of permeation and the unknown relation of keto-enol form of the pyruvic acid used.

Dr. Goddard: From your remarks I assume that the pyruvate we used must have been largely in the keto form. The experiments indicate that the activated spores poisoned with NaF are permeable to the pyruvate used, and that CO_2 is liberated from it at least as rapidly as is CO_2 of natural fermentation. If there is a permeability difference between dormant and activated spores, the dormant spores, in contrast to the activated spores, may be impermeable to keto pyruvic acid.

Dr. Nord: I might add that even if you keep ordinary pyruvic acid in a bottle at room temperature you might observe after the lapse of a short time a little pressure in the container. This is possibly due to liberated carbon dioxide; this has nothing to do with the keto-enol transformation. Therefore it would be desirable to know whether the pyruvic acid which you used contained a little, even a very little, quantity of acetaldehyde, formed during heating.

Dr. Goddard: The pyruvate was not heated during the activation of the spores. 1 ml. of cooled activated spores and 1 ml. of M/20 pyruvate were added to the respirometer vessels. The same pyruvate was used with dormant and activated spores. The experiments with dormant spores are adequate controls to show that no measurable non-catalytic decarboxylation of pyruvate occurred in these experiments.

Dr. Nord: I still think there might be a possibility of explaining your difficulties or differences by the relation of the keto and enol form or the difficulty of the former to permeate.

Dr. Goddard: That may be true, but it would mean a change in permeability on activation of the spores.

Dr. Baumberger: What pH did you use in studying the effect of pyruvic on heated spores?

Dr. Goddard: We worked with unneutralized pyruvic acid, with half neutralized acid (pH = 2.5) and with sodium pyruvate at pH 5.9. Best results were obtained at half neutralization.

Dr. Baumberger: Müller and I made a reinvestigation of keto-enol tautomerism of pyruvic acid and found 50 per cent keto and 50 per cent enol at pH 5.8, and the keto form increased very rapidly with decrease in pH; so that at pH 3.5 you would have only a very small amount, about one per cent, of enol and 99 per cent of keto. This might account for the slow reaction at that pH.

Dr. Goddard: We are not dealing with a slow production of CO_2 from pyruvate. In the dormant spores no measurable CO_2 is formed ($Q^N_{CO_2} < 0.03$). In the activated NaF poisoned spores the rate of CO_2 formation is at least as rapid as natural fermentation in these organisms, at least 160 times higher than any possible CO_2 evolution in the dormant spores.

Dr. Baumberger: There is also the possibility that the pH inside the spore might be quite different, and that after the penetration of the keto form it would be converted to the enol form and react in that form.

Dr. Goddard: That would appear to be most likely. There is no doubt of the rapid penetration and decarboxylation of added pyruvate in activated spores; the whole question is whether pyruvate fails to penetrate dormant spores, or whether it penetrates and fails to undergo decarboxylation.

Dr. Hogness: The very high heat of activation you get for the activation and the deactivation suggests there is a protein involved. I know of no such very high heats of activation except in the presence of a protein.

Dr. Goddard: That is a reasonable suggestion; the deactivation may well be the return of a denatured protein to native protein.

Dr. Lipmann: I think it would be interesting to use an ultramicro method newly developed by Linderstrøm-Lang and co-workers for estimating cozymase and cocarboxylase with diver-manometers. Extremely small amounts of material are needed. I might point out that in the Linderstrøm-Lang method washed yeast is used to determine cocarboxylase and cozymase; one can determine it independently of the enzymatic system of cells such as yours.

Dr. Goddard: I think your suggestion is an excellent one, and I hope to be able to carry it out during the next year.

Dr. Cori: May I ask how completely the fermentation scheme of the spores follows the Meyerhof scheme?

Dr. Goddard: We have no evidence that the fermentation mechanism in the spores follows the Meyerhof scheme; the Meyerhof scheme was

merely accepted as a working theory on which to plan experiments.

Dr. Burk: Your data display a typical Meyerhof quotient; that is to say the value of Q_{CO_2} obtained anaerobically happening to be the same as the Q_{O_2} means that you have a typically Meyerhof oxidation quotient of 3, and I should say that indicates quite possibly one of these fermentation schemes.

Dr. Goddard: Unfortunately the R.Q. of activated spores has not yet been measured. As soon as that is determined the Meyerhof quotient may be calculated.

Dr. Burk: I understood you to say you had an R.Q. of 1.

Dr. Goddard: The dormant spores have an R.Q. of 0.85.

Dr. Burk: Which are the spores that give an aerobic Q_{O_2} of 8?

Dr. Goddard: The activated spores have a Q_{O_2} of above 8.0 and a $Q^N_{CO_2}$ of the same value.

Dr. Burk: About five years ago Brunstetter and Magoon obtained a very striking case wherein the addition of one particular substance to *B. mycoides* spores caused germination to set in immediately, as measured both by microscopic changes and also by the Q_{O_2}. In about 10 minutes a remarkable increase in metabolism measured occurred. The causative substance was peptone.

Dr. Goddard: I do not know the work of the authors. In the case of *Neurospora* the organism has not yet been successfully grown on synthetic media. However, the activated spores will germinate in distilled water or inorganic buffers, and grow for several hours. The dormant spores will not germinate (without heat activation) in any media tried to date, which include malt extract, beerwort, peptone, etc. Nor have I been successful in the attempts to induce germination with thiourea, thiocyanate and ethyl chlorohydrin. It still may be possible to induce germination with the proper factor, if we can find it.

Dr. Burk: What happened to the methylene blue added to the very black spores?

Dr. Goddard: It is apparently on the surface, though it may have penetrated the spores.

Dr. Burk: But when you add, say, acetone or alcohol would most of the methylene blue be eluted back?

Dr. Goddard: No quantitative estimation was made of the extracted methylene blue; from the color I believe that most of it was recovered.

Dr. Burk: It seems to me that in this rather simple case it would be highly pertinent to determine where the methylene blue goes. I suspect you might get it all back by adding alcohol or some other solvent.

Dr. Goddard: The experiments would not be difficult to carry out.

Dr. Melnick: Am I right in believing you said there is no evidence for the respiratory function of carboxylase or of cocarboxylase?

Dr. Goddard: I hope I did not make such a statement. As far as I am aware the evidence for the respiratory function of carboxylase (as contrasted to cocarboxylase) is not clear; though it has been suggested that in some cases all the CO_2 of respiration may arise by the decarboxylation of 3-carbon or 4-carbon keto acids.

Dr. Melnick: In view of your statement that there is no available evidence for a catalytic decarboxylation taking place in respiration, I wish to mention some work involving cocarboxylase. Lipmann has shown that cocarboxylase is the coenzyme necessary for the oxidative decarboxylation of pyruvic acid by *B. Delbrückii*. Barron extended this observation when he found that cocarboxylase must be present for both the oxidative decarboxylation and dismutation of pyruvic acid to proceed in a variety of bacteria and in animal tissues. The observation by Stern and myself that reduced cocarboxylase is as active as the oxidized form in the pigeon and in the yeast test system further suggests a respiratory function for cocarboxylase. Barron was able to effect the reoxidation of the reduced thiazol portion of the molecule by the use of hemochromogens or the cytochrome system. That reduced cocarboxylase is oxidized *in vivo* by the cytochrome-cytochrome oxidase system, with perhaps the intermediation of a flavoprotein, is very probable in view of Barron's observation that the oxidative decarboxylation of pyruvic acid in bacteria is inhibited by cyanide, provided that the respiration proceeds through the cytochrome system in these organisms. In yeast, oxygen does not appear to be the final hydrogen acceptor in the reoxidation of dihydro-cocarboxylase, for the decarboxylation of pyruvic acid takes place at the same rate under aerobic and anaerobic conditions. This is to be expected as anaerobic fermentation takes place in yeast at a rapid rate.

Dr. Goddard: I think we must clearly distinguish between the carboxylase system yielding acetaldehyde from pyruvic acid and aerobic decarboxylation which yields acetic acid. The same coenzyme functions in both systems, but as far as I know, there is no evidence that carboxylase (as usually defined) participates in aerobic decarboxylation. We have no evidence as to whether it is the protein or the coenzyme which is activated during heat treatment. If it is the coenzyme, it may be functioning in a quite different locus aerobically and anaerobically. This might explain the agreement between activation of fermentation and respiration.

Dr. Nord: Have you tried ethylene and acet-

ylene? These substances increase permeability, as has been shown by Nord and Franke (Protoplasma *4, 547, 1928*) and Nord (Angew. Chemie *42, 1022, 1929*).

Dr. Goddard: No, we have not tried either ethylene or acetylene. Your suggestion is an excellent one, and should not be difficult to carry out.

Dr. Stannard: How sure are you that this small amount of CO_2 produced in activation is truly CO_2 and not liberated, let us say, by some acid formation from the pre-formed CO_2?

Dr. Goddard: We determined CO_2 production in parallel vessels, in phosphate buffer pH 5.9 and in bicarbonate buffer equilibrated with 5 p.c. CO_2. The CO_2 production was the same in both cases, indicating no acid production.

Dr. Salomon: When you add methylene blue to mammalian erythrocytes the cells show an increased respiration, as has been shown by Harrop and Barron. When you add methylene blue to cytolyzed cells no stimulation of respiration takes place. It has been shown by Warburg that the cell structure in the experiments mentioned can be replaced to some extent by hexosemonophosphate. In the presence of hexosemonophosphate, methylene blue produces an increase in oxygen uptake in hemolyzed cells as well as in intact non-nucleated erythrocytes. I wonder whether a similar experiment carried out with your material would overcome your difficulties with methylene blue?

Dr. Goddard: Spores cannot be cytolyzed because of the presence of a rigid cell well. It is possible in many plant cells to destroy the semipermeable membrane with toluene, saponin, etc. This was tried with spores and methylene blue added, with no effect. Though hexosemonophosphate was used in intact cells, we have not added methylene blue and hexosemonophosphate simultaneously. This experiment might yield interesting results.

Dr. Barker: I am interested in your return to the unitary theory. With the three types of mammalian muscle with which we worked we were able to separate glycolysis and respiration. However, with testis this separation apparently cannot be attained, as the smallest amount of iodoacetic acid or of any other material which will decrease anaerobic glycolysis will inhibit respiration to an equal or even greater extent.

Dr. Goddard: We have no convincing evidence of a common pathway of respiration and fermentation in *Neurospora*; but the close agreement in the activations of respiration and fermentation appears to be too close to be fortuitous. Though the iodoacetate work on muscle seems to have dealt a death blow to the unitary theory as fas as that tissue is concerned, we are not justified in generalizing the results and discarding the unitary theory for all tissues and organisms. Your results with testis, and Turner's results on carrots, strongly suggest a unitary theory for those tissues, or perhaps it is better to say that the point of divergence of fermentation and respiration differs from tissue to tissue.

Note added Aug. 25, 1939

In the discussion of the paper, Burk called attention to the desirability of knowing the R.Q. of germinating spores. The R.Q. has been determined since the presentation of this paper and the values obtained are: dormant, 0.85; activated, 1.70; germinating, 1.01. These are the average values of 3 separate determinations. The activated value changes with time, gradually merging in to the value of the germinating spores. The activated R.Q. is calculated from total CO_2 and O_2 in a period of from $\frac{1}{2}$ - $1\frac{1}{2}$ hours after activation. The high R.Q. shows that aerobic fermentation occurs in activated spores and the $Q^{air}_{CO_2}$ of fermentation $= 4.70$. The Q_{O_2} for the same period was 5.37. The anaerobic CO_2 was not run simultaneously, but it is usually very similar to the Q_{O_2}. This indicates a very low Meyerhof quotient.

A TENTATIVE PICTURE OF THE RELATION BETWEEN PHOTOSYNTHESIS AND OXIDATION REACTIONS IN GREEN PLANTS

Hans Gaffron

A green plant burns in respiration the organic substances which have been built up previously by photosyntheses, mainly carbohydrates. It is an old problem whether respiration and photosynthesis inside a chlorophyllous cell are opposed to, but independent of, one another, or whether there exists a hidden connection between both reactions. A related, but different question is whether molecular oxygen has any direct influence on the mechanism of photosynthesis.

The purpose of this communication is to present for discussion an answer to these questions on the basis of old well known facts and those which have appeared recently. On the basis of this experimental evidence, the answer which we are prepared to give today is: the uncoloured part of a plant cell contains a respiratory system which has no connection with the chlorophyllous mechanism. But the latter consists of a system of catalysts which partly may bring about very rapid oxidation reactions, different from the normal respiration. In addition the photosynthetic apparatus, working as an oxido-reduction system, is influenced by molecular oxygen in a manner reminiscent of the effect of oxygen on fermentation known as the "Pasteur reaction".

The highest efficiency of photosynthesis has been found under circumstances where respiration surpasses assimilation, i.e. at rather low light intensities. What was actually measured was the respiration in light and in darkness (net O_2 consumption or CO_2 production). The highly important data on the efficiency of photosynthesis depend therefore on exact knowledge of the oxidation reactions going on in the plant, and to some extent on the method of how the measurements of respiration are used to compute the real photosynthesis.

At high light intensities the rate of carbon dioxide assimilation exceeds that of the dark respiration, often to such an extent that the latter can be neglected. But here the question arises whether a strong photosynthetic activity may not speed up respiration during the period of illumination. Van der Paauw (25) has reported phenomena which could be interpreted in terms of a respiration accelerated in the light.

For theoretical reasons Franck and Wood (11) postulated a few years ago that light saturation in photosynthesis is produced by oxidation reactions which increase with intensity until they eventually counter-balance the reduction processes.

Warburg (31) observed a strong depressing effect of oxygen upon photosynthesis which decreased on one hand with the partial pressure of this gas, and on the other hand with the light intensity, i.e. the rate of carbon dioxide assimilation. Warburg's explanation, that this was probably due to an oxidation of the first products of photosynthesis, has been discussed as the most likely one by Briggs (4) who had written even earlier, "We are almost entirely ignorant of the products of photosynthesis intermediate between carbon dioxide and sugar. It is quite possible that some of the products preceding the ordinary sugars may be oxidised more readily than these latter. So it will be seen that there is little ground, except convenience, for assuming that respiration in assimilating cells is the same as when they are darkened."

This question has been discussed for many years without a generally accepted solution, because, for one thing, it was only possible to study respiration separated from photosynthesis, but not photosynthesis without the interference of respiration. Experiments with specific poisons like cyanide have always indicated that photosynthesis was more easily inhibited than respiration. But recently it has been found that this is not invariably so. In certain strains of the alga *Scenedesmus* the respiration is more sensitive to cyanide than is photosynthesis. One of the first experiments to be performed with this plant, therefore, was to ascertain to what extent the inhibition of oxidation reactions, which might have intermediate products in common with photosynthesis, would affect the reducing mechanism (13).

Table I shows that an inhibition of respiration of about 96 p.c. makes an enormous difference in the actual experimental readings. But the computed "true" photosynthesis remains unchanged. There is no indication for the existence of a special "compensation" reaction different from or interfering with normal photosynthesis. Recently McAlister (21) has developed a sensitive method for following the carbon dioxide exchange of wheat plants, whereby changes in rates of photosynthesis and respiration can be followed. Summarizing his results he writes: "The immediate appearance of respiration at the termination of illumination with a rate equal to that maintained before illumination together with its independence of light intensity lead to the conclusion that light has no direct effect on respiration". Exactly the same results have been reported by Emerson (10).

Thus it appears to be justified more than merely from "convenience" (to refer to Briggs) to assume that respiration in assimilating cells is the same as when they are darkened.

But there remained unanswered the problems

TABLE I

Independence of assimilation and respiration with respect to treatment with HCN. 0.090 cc. of cells of *Scenedesmus* suspended in 5 cc. M/40 phosphate solution. pH 5.9; temperature 21° C. Gas phase, air with 5 p.c. CO_2. Low light intensity, 400 Lux.

	Respiration and photosynthesis in mm.^{3} O_2 absorbed or produced			
	control	M/50,000 HCN	M/10,000 HCN	M/2,000 HCN
Dark 20 min. (respiration)	—54	—27	—13	—2
Light 15 min., dark 5 min.	—23	+11	+18	+29
Photosynthesis in 15 min. corr.	+31	+38	+31	+31

connected with Warburg's observation of the inhibiting influence of oxygen of higher partial pressure and those of Boussingault, of Pringsheim, and of Willstätter and Stoll (33), that the presence of very small amounts of oxygen appear to be indispensable for photosynthesis. If respiration cannot, as indicated above, be made responsible for these effects, one has to assume that the assimilatory mechanism itself possesses some affinity to molecular oxygen. Kautsky even goes so far as to state that all the light energy used in photosynthesis is first transmitted to molecular oxygen, because he observed that the fluorescence of chlorophyll in living cells is quenched by oxygen. There is no experimental evidence to support this rather paradoxical assumption.

The obvious experimental approach to these problems was to re-investigate photosynthesis under anaerobic conditions. Willstätter and Stoll had found that a very long anaerobiosis resulted in a complete inhibition which vanished only after prolonged irradiation. These experiments had to be repeated with plant material unlikely to be irreversibly damaged by absence of oxygen. Strains of algae of the genera *Scenedesmus, Chlorella,* and *Coccomyxa,* which easily survive an anaerobiosis lasting from 12 to 24 hours, were used. To get specific effects on photosynthesis due to lack of oxygen it was not sufficient to use the strongest oxygen absorbing chemical in an atmosphere of nitrogen for the purpose of eliminating all the oxygen which might have been bound in the plants in a "dissociable" form. If this anaerobic treatment was thorough-going but short the difference, if any, in the metabolism of the treated plants compared with the controls in air was not large enough to be recorded by the manometric method used. But if the plants were submitted to anaerobic conditions in the dark for several hours, a very conspicuous change in the metabolism after subsequent exposure was the result. Apparently it was the accumulation of fermentation products or the adaptation of enzymes or changes of permeability or, more probably, the combination of several of such reactions, which was responsible for the results observed. These results varied somewhat with the strain of the algae, the methods of cultivation, and the composition of the medium in which the algae were suspended during the experiments. We need to give here only a few examples chosen from the material published elsewhere (12, 14, 16). It is known that under normal aerobic conditions photosynthesis of plants kept in the dark appears to be inhibited during the first minutes of subsequent illumination. Reduction of carbon dioxide as well as the production of oxygen, starting at a low rate, will rise to the more or less stationary rate in the course of 2 to 5 minutes. This so-called induction period does not appear in plants which have been incubated anaerobically for several hours. If the light is turned on after such treatment carbon dioxide reduction begins immediately. But during the first minutes there is a remarkable change in the ratio of carbon dioxide reduced to oxygen liberated.

This ratio, the assimilatory quotient, was always supposed to be unity in photosynthesis, and from this apparently unalterable relation between the volumes of the two gases conclusions as to the mechanism of the process have been frequently drawn.

Tables II and III demonstrate that the assimilatory quotient may vary considerably and may even lose its meaning completely because carbon dioxide is produced, instead of consumed, by the cells during the first minutes of illumination (Table III). A value of —1 for the assimilatory quotient, therefore, indicates only that the process has reached a stationary state where all reactions involved are in equilibrium with one another.

A satisfactory explanation for the abnormal reaction is the assumption that after a prolonged anaerobic period there is much combustible sub-

TABLE II

Rate of photosynthesis and the assimilatory quotient during a sequence of aerobic and anaerobic periods. *Scenedesmus* spec. D 1, 0.060 cc. of cells in 6 cc. and in 3 cc. of suspension. Cells grown four days in medium with 1.5 p.c. glucose suspended in phosphate solution with 0.5 p.c. glucose. Temp. 20° C. Gas phase, O_2 and N_2 with 4 p.c. CO_2. High light intensity.

	Rate of photosynthesis in mm.^{3}/2 min.						
	Aerobic period		Anaerobic period		Aerobic period		
Dark time preceding illumination	2 hr.	20 min.	15 hr.	4 min.	1 hr.	15 min. light	
Gas phase	96 p.c. O_2	0.2 p.c. O_2 in N_2	N_2	N_2 (0.2 p.c. O_2)	96 p.c. O_2	96 p.c. O_2	0.2 p.c. O_2 in N_2
O_2 produced	+24	+42	+24	+17	+23	+44	+50
CO_2 consumed	−20	−35	−12	−10	−26	−44	−50
Assimilatory quotient	−1.2	−1.2	−1.7	−0.9	−2.0	−1.0	−1.0

TABLE III

Photochemical production of CO_2 during the induction period after an anaerobic dark time. *Scenedesmus* spec. D 1, grown autotrophically. 0.048 cc. of cells suspended in 3 cc. of 0.01 M $NaHCO_3$. Temp. 20° C. Incubated for 17 hours in an atmosphere of N_2 with 4 p.c. CO_2. Vessel I without, vessel II with 0.2 cc. of "Oxsorbent" ($CrCl_2$ sol.) in side bulb for absorption of oxygen.

	Vessel I	Vessel II		
Total volume (cc.)	15.4	15.0		
Liquid phase (cc.)	3.0	3.2		
Vessel constants				
K_{O_2}	1.16	1.10		
K_{CO_2}	1.41	1.37		
	Manometric pressure changes		Assimilatory quotient calculated	
	O_2 and CO_2	CO_2 only		
2 min. light + 2 min. dark	+7	+5	+2 mm.^{3} O_2 +7 mm.^{3} CO_2	A.Q = +0.29
5 min. dark	+0.5	−0.5		
8 min. light + 5 min. dark	+6	−29	+41 mm.^{3} O_2 −40 mm.^{3} CO_2	A.Q = −1.03

stance in the neighbourhood of the reducing mechanism. The photoperoxides, precursors of molecular oxygen in the process of photosynthesis, would then react with hydrogen donors instead of liberating free oxygen. If these combustible substances or hydrogen donors were carbohydrates the only observable effect would be an apparent inhibition of photosynthesis whenever such internal photoxidations occurred at a high rate. Fortunately for our analysis of photosynthesis algae grown in media containing 1.5 p.c. glucose display a respiratory quotient which is not unity but averages 1.4 and often approaches 2 (15). For each volume of oxygen used in respiration one and one half times, or even twice, the amount of carbon dioxide is formed. With hydrogen donors giving high respiratory quotients any photoxidation reaction accompanying photosynthesis must result in a considerable disturbance of the assimilatory quotient, thus revealing the existence of such "back reactions."

There are several observations which show that the assumption of reactions between photoperoxides and combustible substances is correct. We will mention only one more. If it were possible to make the photoperoxides react with a hydrogen donor yielding no carbon dioxide, the assimilatory quotient should shift to the opposite direction. The production of oxygen should be diminished in comparison with the absorption of carbon dioxide.

Some strains of the alga *Scenedesmus* when treated with molecular hydrogen under anaerobic conditions are capable of adapting themselves to utilising molecular hydrogen in connection with oxidation reactions. In other words they contain or develop hydrogenase, a type of enzyme found hitherto only in bacteria. With these algae it is possible to separate carbon dioxide reduction entirely from oxygen liberation and prove that both reactions are only loosely connected (16). The one is not invariably the consequence of the other. In 1887 N. Pringsheim (cited after Willstätter and Stoll, 32) had already reached the same conclusions from his observations on the behavior of cells of *Chara* in hydrogen. In comparison with the extended investigations of Willstätter and Stoll on the influence of anaerobiosis on photosynthesis Pringsheim's views appeared not so well founded and gradually fell into oblivion. Fig. 1 gives an example of how the reduction of carbon dioxide with molecular hydrogen can be demonstrated manometrically. After several hours in the dark in contact with pure hydrogen a suspension of algae will commence to absorb hydrogen when illuminated together with those amounts of carbon dioxide which have been formed by fermentation. The rate of this reaction will gradual-
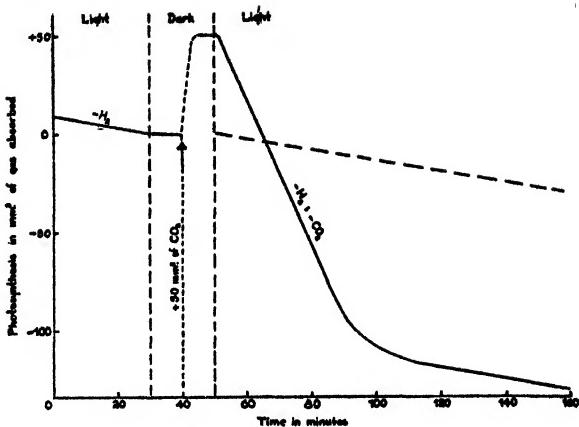

FIGURE 1

ly drop till a rather constant slow rate of hydrogen absorption in continuous illumination has been reached. If the suspension is darkened scarcely any gas exchange can be observed. Now a certain amount of CO_2 is introduced, for instance by adding a little dissolved sodium carbonate (Na_2CO_3) to the acid cell suspension (dotted line in Fig. 1.). 50 mm.[3] of CO_2 added in the dark will bring about a rapid absorption of more than 150 mm.[3] of gas on subsequent illumination. Corrected for the basic hydrogen absorption which in all experiments was found to continue, the result agrees well with the equation

$$CO_2 + 2H_2 + H_2O \rightarrow CH_2O + 2H_2O .$$

It is certainly not self-evident that phenomena so different as the photochemical utilisation of molecular hydrogen and the photochemical production of carbon dioxide, as shown for instance in Table III, should result from the same type of reaction. On the one hand it is possible to show that the reaction with hydrogen in *Scenedesmus* is inhibited to a great extent by the presence of sugar or yeast autolysate in the medium. This, of course, is in favor of our assumption. We may say that one hydrogen donor is replacing the other. On the other hand we must ask why internal oxidation reactions should occur at a high rate under just those anaerobic conditions which in many instances appear to accelerate normal photosynthesis. I mentioned in the beginning Warburg's observation that lowering the oxygen partial pressure will increase the rate of photosynthesis. In Table II we see that a change from 96 p.c. O_2 to 0.2 p.c. O_2 brings about a rise of 75 p.c. (first aerobic period). It is somewhat plausible that at high light intensities the concentration of sugar becomes so high as to enforce a back reaction in the sense of Franck and Wood. But it appears as a contradiction that the very process

from which nearly all organic substance originates should generally be linked with breakdown reactions of the same order of magnitude, sometimes capable of completely inhibiting oxygen production. Furthermore, we may ask how the photosynthetic mechanism saturated during anaerobiosis with glucose and fermentation products can recover from the contended photochemical oxidations particularly if simultaneously even more glucose is produced. A satisfactory answer, I believe, can be given by considering more closely the conditions ruling the phenomena we have been discussing. The capacity of the green algae to utilise hydrogen depends on the preceding anaerobic incubation; so does the appearance of a disturbed assimilatory quotient, and it was observed several years ago that prolonged anaerobic incubation renders the photosynthetic mechanism of the plant sensitive to carbon monoxide which only under these circumstances acts as an inhibitor (12). All three phenomena vanish either with the introduction of oxygen from outside or the eventual liberation of oxygen inside the cell. The rate of reduction of CO_2 with H_2, for instance, will rise in proportion to the intensity up to a certain intensity. If this limiting intensity is surpassed, the absorption of hydrogen will soon stop, giving way to normal photosynthesis with the production of molecular oxygen. This remarkable change takes place before any considerable oxygen partial pressure has been built up outside the cell. We have to conclude therefore that oxygen in small amounts has a regulating influence on the course of the reactions taking place in the assimilatory system. Six years ago G. E. Briggs wrote: "The data for photosynthesis are so numerous that the time seems ripe for the more precise formulation of theories, many of which have been but vaguely suggested and rarely put to a quantitative test. The process of formulation, testing, and rejection or refinement must necessarily be tedious for such a complicated biological reaction."

With the additional data accumulated since that time a comprehensive picture of the various reactions in photosynthesis is even more urgent. For the part played by oxidation reactions the available data are sufficient to give at least an outline. If the different observations about which we have been talking are assembled in the form of the diagram presented in Fig. 2, such an attempt should not be called a theory. There is no doubt but that the final scheme of photosynthesis will look different and more complicated when all the intermediate steps and the nature of all the catalysts involved are known. The diagram has not been drawn with the hope that it might survive many years of experimental progress, but that it should be used as a guide in discussing a large variety
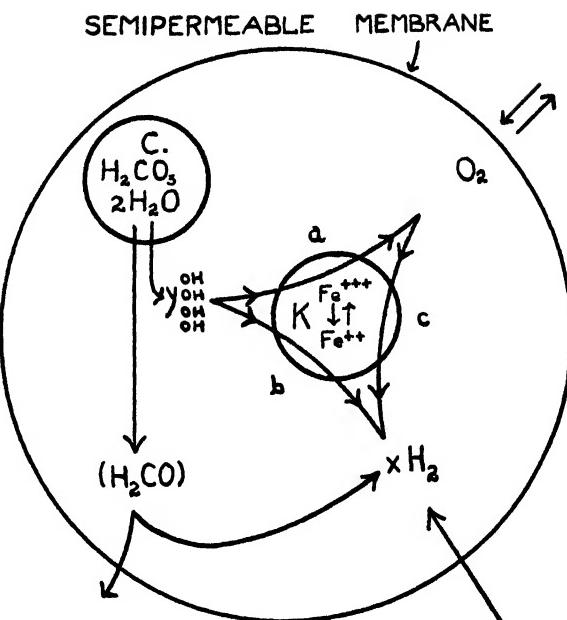
FIGURE 2

of different observations which are difficult to keep in mind without such a device. Its deliberate simplicity will probably challenge disproof or confirmation by new experiments and that is the way such a diagram can be expected to be useful.

The center of our picture is held by a ring representing the "photocatalase" or the "oxygen liberating system." The ring at the left represents the complex formed by carbon dioxide, water, chlorophyll and unknown catalysts. The problems of how four quanta are collected and of how the carbon dioxide is actually reduced are both outside the scope of this communication. Also the extent to which the interpretation which we are going to present will fit in with the theoretical deductions of Burk and Lineweaver, Franck and Herzfeld, Wohl, Ornstein and others has yet to be developed.

The photochemical process yields the following products: carbohydrate of an unknown constitution and what is left of 2 molecules of water when the hydrogen has been taken away which is necessary to transform carbonic acid into sugar—namely 4 hydroxyl groups.

It is well known that all the theories of photosynthesis presented between 1926 and 1936 assembled the 4 hydroxyl groups in the form of hydrogen peroxide which was supposed to be split subsequently by catalase.

Here we collect the hydroxyl groups in a complex $Y(OH)_4$ which for convenience I shall call the intermediate photoperoxides. It is irrelevant whether $Y(OH)_4$ consists of one or more molecules. It is certain that $Y(OH)_4$ is not hydrogen

peroxide because the capacity to split H_2O_2 can be separated in a green alga from its capacity to produce oxygen in photosynthesis, and because small amounts of H_2O_2 are a specific poison for the dark reactions in photosynthesis it becomes clear that the photocatalase must be different from ordinary catalase (13). If we take into account the parallelism in the behavior of catalase and of the enzyme acting in photosynthesis in the presence of such substances as HCN, NH_2OH, N_3H, H_2O_2 and CO it seems safe to postulate that the oxygen liberating enzyme in green plants differs from the well known catalase only in the protein constituent determining its specificity. The most striking resemblance is the reaction with carbon monoxide. Neither the action of catalase nor photosynthesis appeared to be inhibited by carbon monoxide. But Keilin and Hartree (19), working with a pure preparation of the enzyme catalase, discovered that whereas catalase did not react with carbon monoxide ordinarily, it combined with carbon monoxide under specific conditions. These were: absolute absence of oxygen, presence of complex-forming substances like hydroxylamine or glutathione and a little peroxide necessary to reduce the iron of the catalase from Fe^{+++} to Fe^{++}. Under the same or at least very similar conditions the action of carbon monoxide on photosynthesis has been previously observed. A long anaerobic dark time was essential so that everything in the cell appeared to be reduced.

Recently it has been found by Agner (1) that the highest activity of catalase was observed with those preparations which contained a certain amount of copper bound to protein. It is therefore very interesting that a chemical analysis of the chloroplasts by Neish (23) revealed that they contained not only iron but also relatively large amounts of copper. According to Neish, the fact that Fe and Cu show the same localization (in the chloroplasts) supports the view that some oxidative reactions are centered in the chloroplasts, where their catalytic action is particularly required.

If we turn now to the activities of the oxygen liberating enzyme, we have to consider first the splitting of the photoperoxides $Y(OH)_4$ (Fig. 2, reaction a). Iron-porphyrins are oxidation catalysts, and catalase, as Keilin and Hartree (19) and Stern (30) have shown, is no exception. Its action, according to these authors, consists in an oxidation of H_2O_2. All the oxygen is liberated and the iron in the catalase molecule is reduced to the divalent state. Half the amount of molecular oxygen is afterwards necessary to re-oxidize the iron to re-activate the enzyme. In the absence of oxygen the catalase remains inactive.

Having said so much in favor of the chemical similarities between catalase and the oxygen re-leasing enzyme in photosynthesis it is only logical that we ascribe to the latter the same mode of action.

We assume that $Y(OH)_4$ is split under evolution of the total amount of oxygen. The catalyst at the same time is reduced. But the reaction can continue only if there is sufficient oxygen to re-oxidise the photocatalase. If the conditions are such that the small initial amount of oxygen liberated is used up in other reactions the liberation of oxygen will not continue.[1] Such a type of reaction explains therefore the photosynthetic activity of plants after a prolonged anaerobic period. $Y(OH)_4$ is not decomposed further and remains available for reactions of type b of the diagram, that is, a reduction by hydrogen donors (XH_2). What kind of organic substances may be used as sources of hydrogen depends on the specificity of the dehydrogenases present, and the latter may vary from plant to plant. It is not surprising that only some algae are capable of activating molecular hydrogen. Among the photosynthetic purple bacteria the strains also differ in this respect.

The presence of hydrogen donors and of oxidation catalysts which certainly have an affinity for molecular oxygen call for the existence of a reaction of type c. This would mean a respiration directly connected with the assimilatory mechanism. Reaction c will explain why oxygen has such a strong depressing effect on photosynthesis at high light intensities and further why those algae which utilise hydrogen for the reduction of the photoperoxides may catalyze the oxyhydrogen reaction in the dark. Whether the whole system of the oxygen liberating enzyme or only part of it will, when in the reduced state, participate in reactions b and c remains an open question, of course.

Those familiar with the advances in our knowledge of the metabolism of the photosynthetic purple bacteria will have noticed that our scheme has been drawn along lines similar to van Niel's attempt to bring photosynthesis in plants into accord with the photochemical reactions in bacteria (24). van Niel remarked four years ago that in the bacteria which use hydrogen donors other than water these hydrogen donors might not reduce CO_2 directly but possibly by way of the intermediate compounds resulting from a photochemical reaction between carbon dioxide and water. That our diagram will serve to explain the metabolism of purple bacteria as satisfactorily as that of green plants is also revealed by a comparison with the new findings of Nakamura (22). This author

[1] Blinks and Skow (3) found that after long anaerobic dark periods, followed by an apparent inhibition of photosynthesis, a small amount of oxygen as well as of carbon dioxide is produced during the first seconds of illumination.

observed that the oxygen uptake, of purple bacteria in the dark was greatly diminished if the bacteria were irradiated in the presence of CO_2. In our terms Nakamura observed a competition of reactions b and c for the available hydrogen donors.

According to our picture the difference between carbon dioxide assimilation in purple bacteria and in plants lies in the energy content of the photoperoxides $Y(OH)_4$, which, in bacteria, using the smaller energy quanta absorbed by bacteriochlorophyll, is too small for reaction a, the liberation of free oxygen.

So far our diagram accounts for the type of reactions observed in photosynthetic organisms but not at all for the amazing efficiency of reaction a, the liberation of oxygen in green plants. The first products of photosynthesis should be consumed by reactions b and c at such a rate as to make any real accumulation of synthesized organic material improbable. A solution of this problem appears difficult in terms of specificity of catalysts, concentration of reactants or reversibility or inactivation of enzyme reactions.

We must look for an additional device which will protect the newly formed organic compounds from their immediate destruction by the photoperoxides. A new factor has to be introduced for a proper explanation, a factor belonging to that class of phenomena which are generally collected under the term "permeability". There is sufficient experimental evidence for the existence of a membrane or interface encompassing the assimilatory apparatus, to support the hypothesis that such a membrane may be an active and necessary part of the mechanism of plant photosynthesis; for instance: (1) sensitivity of photosynthesis to mechanical injuries of the cell; (2) effect of potassium salts on potassium-deficient cells, which consists in an increase of photosynthesis with a simultaneous diminution of respiration (Pirson, 26, 27); (3) absence of potassium, which constitutes 20 p.c. of the ash of entire leaves, from the chlorophyllous organs (chloroplasts or grana) (Neish, 23); (4) swelling of the chlorophyll granulae when suspended in distilled water without rupturing even after several weeks (Neish, 23).

A protective separation of the products of photosynthesis by means of a special interface or membrane is an assumption quite consistent with the experiments reported. It is known that "permeability" in living cells may change considerably with the presence or absence of oxygen. I refer to the work of Dixon (7), Smythe (29), Lundegard (20), Hoagland and Steward (18). References to work on the subject of anaerobiosis and permeability may also be found in a new review by Quastel (28).

In our diagram the large ring represents the interface which separates the assimilatory mechanism from the rest of the chloroplast. For many reasons, which we cannot discuss here, it has long been claimed that the chlorophyll, or the photochemically active complex, is attached to some structure of the cell. We may believe that this structure and our membrane are identical. The oxygen liberating enzyme, on the other hand, is in all probability moving freely in the medium filling the assimilatory chamber. This can be deduced from experiments in which the photocatalase was irreversibly inactivated for the most part by hydrogen peroxide. Yet the remaining active fraction was able to decompose all photochemical substrates provided these were formed at a low rate, that is, at low light intensities.

The regulating capacity of the hypothetical membrane is easy to understand. The rate of diffusion (or of transport) varies with the permeability (or the activity) of the membrane and with the concentration and the nature of the organic compounds on both sides. Light respiration occurs inside the chamber in proportion to the concentration of the products of photosynthesis: the sugar and the photoperoxides. (We will assume that all active catalysts are present in abundance.) If the speed of transport of the organic compounds through the membrane is higher than the rate of its production, conditions are favorable for a high efficiency of the synthetic process (cf. ref. 2, 6, 18, 20). This will be the case at low light intensities. At very high light intensities the rate of trespassing the interface is a limiting factor and causes most of the organic substances trapped inside the assimilatory chamber to be burned again.

Under anaerobic conditions the "chamber" is flooded with compounds serving as hydrogen donors. On subsequent illumination these will reduce the photoperoxides as long as aerobic conditions are not restored.

The diagram, as might be pointed out in concluding, is consistent with quite different observations. The so-called induction period, that is, the initial inhibition after a period of darkness under aerobic conditions, may be due to a reversibility of reaction a. In the absence of a high concentration of hydrogen donors, reaction b is too slow to keep K and Y in an active state. K and Y become oxidised and on subsequent illumination the greater part of Y is not ready to take over the hydroxyl groups in the photochemical oxido-reduction. Light energy is consumed in this activation of the oxidised system.

If the picture of the oxygen liberating enzyme here presented is true, it would mean that a green plant cell has two different respiratory systems. And it is gratifying to see that such an assumption would offer an explanation for some curious observations on plant respiration which were often

cited fifteen years ago in connection with the cyanide inhibition of respiration in general.

Warburg (31) observed that the respiration of the alga *Chlorella,* instead of being inhibited, was accelerated by small concentrations of cyanide. Later, Emerson (8) and Genevois (17) found this to be true only in the absence of organic substances. If glucose, butyrates or acetaldehyde were present, respiration was 400 p.c. faster than normal. But this accelerated respiration was easily inhibited by cyanide. Genevois (17) has emphasized the possible difference between the nature of the cyanide resistant and the additional glucose respiration in *Chlorella* and similar algae. When the glucose respiration is suppressed by cyanide, aerobic fermentation appears despite the continuous oxygen uptake of the "normal" respiration.

It can be shown that the accelerating influence of the cyanide on this normal respiration is a true catalytic effect and not due to a combustion of the poison (15). Having accepted the idea of oxidation reactions of type c as occurring inside the assimilatory system the behavior of *Chlorella* might be interpreted in the following way:—What we call "normal" respiration of *Chlorella* might be due to reaction c, occurring also in the dark. If cyanide diminishes the affinity of K for Y, inhibiting photosynthesis, it may simultaneously increase the rate of reaction c.

The other reaction (respiration of *Chlorella* in the presence of organic substances, which is poisoned by cyanide) must proceed far outside the assimilatory mechanism. Emerson states that it is sufficient to leave the algae only a few minutes in a medium containing 2 p.c. glucose to obtain an increased respiration, a treatment which produces no difference in the rate of the carbon dioxide reduction.[2]

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[2] Glucose penetrates very slowly into the cells of *Chara* (see Collander and Bärlund, 6). A respiration occurring outside the plasma membrane in a thin surface layer has been assumed by Went (32).

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CARBOHYDRATE AND LIPID ASSIMILATION IN BAKERS' YEAST

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I.

In the experimental attack on the chemosynthetic mechanism both *in vitro* and *in vivo* approaches have been followed. Nishimura (1930, 1931) and Minagawa (1932, 1933) report the extraction of an enzyme from "Peerless" and beer yeast which is capable of polymerizing achroödextrins to a substance giving the starch-iodide reaction. This enzyme was named amylosynthease and was shown to be distinct from amylase by its action toward enzyme poisons and precipitation reagents. Extention of this promising method of attack has come recently from Cori, Schmidt and Cori (1939) and Kiessling (1939). Their purified preparations of phosphorylase bring about the enzymatic synthesis of a high molecular polysaccharide from a phosphorylated monosaccharide (glucose-1-phosphate). According to Kiessling the product formed from glucose-1-phosphate by his preparation of yeast phosphorylase is a polysaccharide indistinguishable from glycogen. The *in vitro* evidence thus indicates that glucose-1-phosphate is the substrate for glycogen synthesis and that "the same enzyme - the phosphorylase - brings about glycogen synthesis and glycogenolysis", oxidative energy being necessary for the phosphorylation of the glucose (Cori, Schmidt and Cori, 1939). For discussions of the transfer of energy between the 'energy yielding' and the 'energy requiring' reactions, see Baumberger, Jürgensen and Bardwell (1933) and Borsook (1935).

Among the more important early *in vivo* investigations on assimilation of carbohydrate and fat in yeast cells might be included the cytological investigations of Errera (1882) establishing the existence of glycogen in yeast and the experiments of Nägeli and Loew (1878) who increased the fat content of yeast and molds by culturing them in a well-aerated medium rich in carbohydrate. Except for the incomplete description of the timecourse of glycogen formation (based on chemical analyses) by Pavy and Bywaters (1907), subsequent investigations on the formation of anabolic storage products of yeast inoculated in a nonnitrogenous sugar medium were concerned mainly with the yields of these products formed after some arbitrary time interval.

Thus, for instance, Laurent (1889) and Kayser and Boullanger (1898) investigated the relation of glycogen formation to fermentation and concluded that glycogen was a transitory substance in the cell intermediate between sugar consumption and alcohol production. Grüss (1903, 1904) adopted this view in his theory of fermentation and postulated glycogen to be a storage material destined for respiration in air and fermentation in the absence of air.

Analytical procedures for the analysis of total carbohydrate, glycogen, yeast gum and total lipids in yeast cells were particularly investigated by McAnally and Smedley-MacLean (1935), Mayer (1923) and MacLean and Hoffert (1923). They determined yields of these anabolic products under several environmental conditions, after an arbitrary period of incubation of the yeast with various substrates.

The "first" step in the utilization of glucose by the intact cell was reported by Willstätter and Rohdewald (1937) to involve the condensation or combination of fermentable sugars into a glycogenlike polysaccharide. The next step was thought by these authors to consist of the cleavage of this polysaccharide by an amylase to active sugar which then combines with phosphate and undergoes the complex chain of fermentation reactions induced by the zymase complex.

Another body of data on assimilation by the living cell has been obtained from gasometric measurements made during the oxidation of known amounts of various nitrogen-free substrates. From the known amount of substrate added, the oxygen consumed and the CO_2 produced it is possible to write a balanced equation and thus deduce the empirical composition of the other product or products. A few examples follow. Meyerhof (1925) found the oxidation of alcohol by yeast to yield a product with the empirical formula $(CH_2O)_n$. Furth and Lieben (1922) concluded that intracellular carbohydrate was formed when lactate was oxidized by yeast cells. Giesberger (1936) with several species of *Spirillum*, Barker (1935, 1936) with the colorless alga *Prototheca Zopfii* and Clifton (1937) with *B. coli* and *Pseudomona calo-acetica*, using the Warburg manometric method, have demonstrated the synthesis of a large percentage of various substrates to cellular material. In all cases that portion of the substrate not burned to CO_2 and H_2O had the empirical composition of a carbohydrate. Recently, Winzler and Baumberger (1938) have demonstrated by gasometric and thermal methods that a large percentage of the glucose which disappears from a yeast suspension is synthesized to intracellular carbohydrate.

The manometric method as applied to assimilation studies in the manner outlined above is obviously open to objections. Burk (1937) has

already given general criticisms in connection with his evaluation of the evidence for the Meyerhof cycle theory of the Pasteur effect. Interpretation of results obtained by this method, without simultaneous chemical determinations of the anabolic products formed, has led to erroneous interpretations of the mechanism of oxidative synthesis (*cf.*, Burk, 1937). Since the method does not enable one to follow the course of the assimilation reaction and does not give precise indications of the nature of the synthesized product or products, its use in assimilation studies is therefore limited.

In the *in vivo* investigations of carbohydrate and lipid assimilation by yeast the dynamic (kinetic) approach has not been employed until recently by Stier and Sprince (1938) and Runnström and Sperber (1938). The view that carbohydrate assimilation in the living yeast cell might be viewed as a reaction proceeding to completion has not been utilized experimentally in the past.

Results from a program of research dealing with the dynamic aspects of carbohydrate and lipid assimilation in the intact bakers' yeast cell will be presented. Chemical methods of analyzing the intracellular anabolic end-products were employed exclusively. Our primary aim in this research was to make a preliminary survey of the course of carbohydrate and lipid synthesis using bakers' yeast under several different physiological conditions with a view toward (1) discovering the technical difficulties involved in following the course of the reaction and in making the large number of chemical analyses required for each experiment; (2) formulating the conditions for a standard assimilation reaction whose properties can then be reproduced under definite, prescribed conditions. We feel that it is important to devise such a standard reaction before proceeding with the modification of the rate and course of assimilation and the experimental attack on the *in vivo* mechanism of the reaction.

II

We have used, for convenience, a commercial strain of bakers' yeast, *Saccharomyces cerevisiae*. It is a pure strain (GM yeast type No. 139) grown in mass culture by the Fleischmann Company under conditions of extraordinary precaution for protection against contamination. The yeast was shipped to us directly from the factory under refrigeration and arrived in our laboratory within 24 hours after separation from the original culture medium. Upon arrival, this yeast was stored immediately in a refrigerator at 5° C. Yeast from the center of the package contained practically no bacterial contaminants. Since the yeast is always washed several times with $M/15$ KH_2PO_4 these contaminants are practically elim-

inated. Furthermore, the experimental conditions usually employed (high yeast concentration, non-nitrogenous sugar medium and an initial pH of 4.5) are unfavorable for the reproduction of the remaining bacteria.

The reaction flask containing the yeast consisted of a round bottom boiling flask bearing a neoprene stopper with an air inlet tube extending to the bottom center of the flask. Air which had been moistened, filtered and brought to the temperature of the bath (25° C.) was bubbled through the suspension in the reaction chamber. The rate of flow of air was kept constant at 100 ml. per minute. The type of flask employed, the position of the air inlet tube and the rate of bubbling are most important for keeping the cells in suspension during the entire course of the reaction. In addition, the flask was also gently shaken during the experiment. This helps eliminate interference from foaming which occurs under certain experimental conditions and which leads to inaccuracies when samples are taken for analysis. Foaming can be completely eliminated by the addition of about 0.05 ml. of olive oil at zero time. We have not as yet determined whether this small amount of olive oil alters the course of carbohydrate or fat assimilation.

At zero time a solution of dextrose in $M/15$ KH_2PO_4 which had been brought to the temperature of the bath and then saturated with the gas mixture employed in the experiment was added to the yeast in the reaction chamber. The final concentration of dextrose in the suspension at zero time was usually 15 per cent.

At various intervals after addition of the dextrose, samples were withdrawn by pipette. When high concentrations of yeast are employed, effervescence of CO_2 may interfere with pipetting. When this condition prevails a portion of the suspension is first transferred to a dry test tube immersed in cracked ice and water contained in a Dewar flask and the sample is then pipetted from this cooled aliquot. Care must be taken to keep all steps in the procedure uniform for all samples taken during the course of a given reaction in order that the same volume is taken each time.

Several methods of stopping the assimilation reaction have been employed: (a) the sample was drained into absolute alcohol, the final alcohol concentration being 75 per cent after the addition of the sample; (b) the sample was added directly to a solution of formalin saturated with salicylic acid, the final concentration of formalin being 8 per cent; (c) the sample was added to an equal volume of distilled water at 2° C. in a centrifuge tube, the yeast then immediately washed 3 times by centrifugation at about 5° C., decanting each time into a Jena No. 4 sintered-glass filter, and

finally transferring the cells quantitatively to the hydrolyzing flasks with 1 N HCl. About 20 minutes elasped between the taking of the sample and the final transfer with 1 N HCl (the cells did not reach a temperature higher than 5° C. during this interval). These flasks were then stored in a refrigerator at 2° C.

The alcohol fixative has the advantage of speed of taking samples during an experiment but has the disadvantage that the fixative extracts some of the lipid material of the cell and probably some of the hexoses. We have not as yet determined the limitations of the formalin fixative. At the moment, the method involving washing the cells with H_2O at 2-5° C. and then immediately suspending them in 1 N HCl saves time in the long run and is not open to the objections raised above. Since the Q_{10} for assimilation has a value of about 6 it can be calculated that during the 3 minutes when the cells are still in contact with a high concentration of dextrose at 5-10° C. there would result only about a 1 per cent increase in total carbohydrate even in the fastest part of the assimilation reaction.

The procedure employed in following the reaction is based on the assumption that the number of cells per volume of sample does not change during the course of the reaction. It is well known that growth by cell division does not take place under these experimental conditions (Geiger-Huber, 1934; Stier, Newton and Sprince, 1939). For suspensions containing up to about 2 per cent of yeast (wet weight) and 5 per cent dextrose we have found no significant change in the concentration of yeast (number of cells per ml. of sample) over the course of 8 hours after adding the dextrose; the values fell within the 6 per cent limit of variation which was taken to be significant when 2,000 to 3,000 cells were counted per sample. For suspensions containing 7-8 per cent yeast and 15 per cent dextrose we have not as yet made cell counts throughout the entire course of assimilation but have observed the change in volume of the entire contents of our reaction chamber during the course of assimilation. A change of about 5 ml. in a total volume of 2,000 ml. occurred at the end of 5 days at 5° C. Further tests of this important point will be made in the near future.

For the analysis of total carbohydrate we have followed the method of McAnally and Smedley-MacLean (1935) with certain minor modifications. The cells were hydrolyzed at 99-100° C. for 3 hours in 1 N HCl while gently shaking the flasks; the total reducing sugar in the hydrolysate was determined by the ceric sulphate method of Miller and Van Slyke (1936). It is realized that some of the steps in procedures of this type are,

in a sense, arbitrary and will need careful study before the final methods giving reliable determinations for a kinetic analysis of the data will be available. For instance, the relation between temperature of the acid hydrolysis and yield of reducing sugar from yeast cells, and duration of hydrolysis and yield has not been thoroughly investigated. For this preliminary survey, we have assumed that the extent of hydrolysis of the carbohydrate, the amount of reducing substance coming from non-carbohydrate sources and the amount of degradation of the reducing substance formed during the course of hydrolysis remains relatively constant in all the samples taken during the course of an assimilation reaction. A completely satisfactory method of analyzing the total carbohydrate content of yeast cells has yet to be developed.

The determination of total lipids in yeast (after the cells have been disrupted by acid treatment) depends likewise upon the method of extraction employed (cf. Smythe, 1938). This matter should also be reinvestigated before proceeding with any final studies of fat synthesis. We have employed the ether extraction method of MacLean and Hoffert (1923) in these survey experiments.

III

Aerobic Assimilation

"Fresh" Yeast

The time-course of the increase in total carbohydrate and in lipids of fresh bakers' yeast (GM type No. 139) is shown in Fig. 1. The cells were suspended in M/15 KH_2PO_4; the initial dextrose concentration was 15 per cent; aerobic conditions were maintained through the entire course of the experiment. At the end of the experiment 0.02 per cent dextrose was still present in the suspension. This is probably a "limiting" concentration of dextrose for assimilation.

At zero time the total carbohydrate content was 0.21 mg. glucose per million cells; at 18 hours, 0.52 mg. per million cells. A kinetic analysis of the declining rate type of curves exhibited beyond the initial "lag" period will be made as soon as we perfect our perfusion technique whereby the dextrose concentration is kept constant throughout the entire course of the reaction and the extracellular by-products of dextrose metabolism are removed from the reaction chamber. Obviously, in order to determine the true asymptotic value for the total carbohydrate stored one must maintain a non-limiting concentration of dextrose throughout the entire course of the reaction, remove all 'inhibitory' by-products and eliminate all technical artifacts from the various procedures involved in following the reaction.
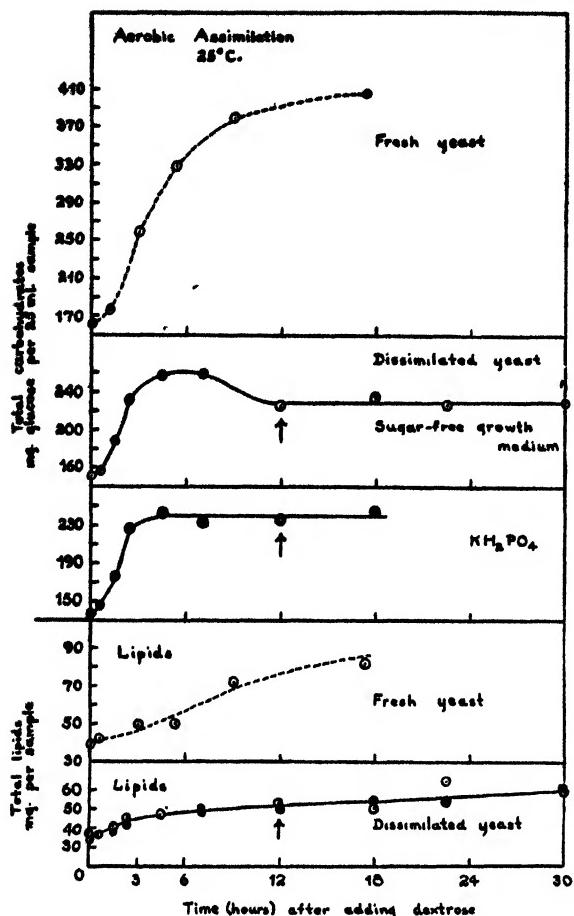
Fig. 1. Time-course of the increase in total carbohydrate and lipids of bakers' yeast under aerobic conditions. Assimilation of the "fresh" yeast was started about 1½ days (at 5-10° C.) after separation from its culture medium. The suspension of fresh yeast contained 780.8 million cells per ml.; the suspension of cells dissimilated in KH_2PO_4, 777.3 million cells per ml., and the suspension of cells dissimilated in sugar-free growth medium, 735.8 million cells per ml. Arrows mark points where all the dextrose had disappeared from the suspension. (Data of Stier and Simonds.)

Dissimilated Yeast

Since the glycogen content of bakers' yeast cells decreases during the interim between separation of the cells from their culture medium at the factory and the beginning of an experiment (see Stier and Stannard, 1936) it was thought advisable to bring the cells to a minimum level of carbohydrate and fat content before starting to assimilate them. At the end of a 3 day period of aeration in M/15 KH_2PO_4 (25° C.), the majority of the cells (ca. 80-90 per cent) of GM type No. 139 undergo no further cytological changes. At this time they present a distinct cytological picture peculiar to this treatment. The nuclear vacuole

becomes much enlarged and appears to be surrounded, as it were, by a rim of cytoplasm; the cells absorb practically no Gram's iodine. Plate counts on these cells show the viability to be unaffected by this treatment. It was felt that this treatment gave a suspension of cells of more uniform morphology since in suspensions of fresh yeast one always finds a variable number of cells of the fully dissimilated type. Furthermore, it was thought that suspensions of these fully dissimilated cells would give a better base line from which to start their assimilation. A sugar-free growth medium (Williams, et al., 1927) was also used for dissimilating fresh bakers' yeast.

Fig. 1 also shows the course of carbohydrate assimilation of cells dissimilated for 3 days at 25° C. in KH_2PO_4 and in the sugar-free Williams' medium. Note the marked reduction in the total carbohydrate assimilated under these physiological conditions. The maximum per cent increase obtained in dissimilated cells is about one-half of the maximum observed for the fresh cells (Fig. 1).

The results obtained from dissimilated cells are of special interest for future work on the mechanism of assimilation. The dissimilated cells have not only lost carbohydrate and fat during the "starvation" treatment but have probably lost a variety of important units of their metabolic machinery. Pett (1936) showed that the lactoflavin content of his laboratory strain of yeast fell from 24 gamma per gram (dry weight) to 6 gamma per gram during 36 hours of aerobic dissimilation in phosphate buffer. We also find that GM yeast has a lower flavin content after it has been dissimilated in KH_2PO_4 or in the sugar-free growth medium. The results presented in Fig. 1 indicate that the vitamin B_2 deficient yeast cells store carbohydrate at about the same rate as the normal cells during the early stages of the process but that carbohydrate storage stops much sooner than in the "normal" yeast. The role of riboflavin in carbohydrate and fat synthesis will receive further study. It is hoped that other types of dissimilation procedures will enable us to prepare cells lacking in various other metabolic elements so that we may test the effect of their absence upon assimilation.

Anaerobic Assimilation

The time-course of total carbohydrate and lipid assimilation under anaerobic conditions is given in Figs. 2 and 3. The data for aerobic assimilation (smaller points) are also included for comparative purposes. The strain of yeast employed and the experimental procedures were the same as in the experiments given above under aerobic assimilation. Anaerobic conditions were maintained
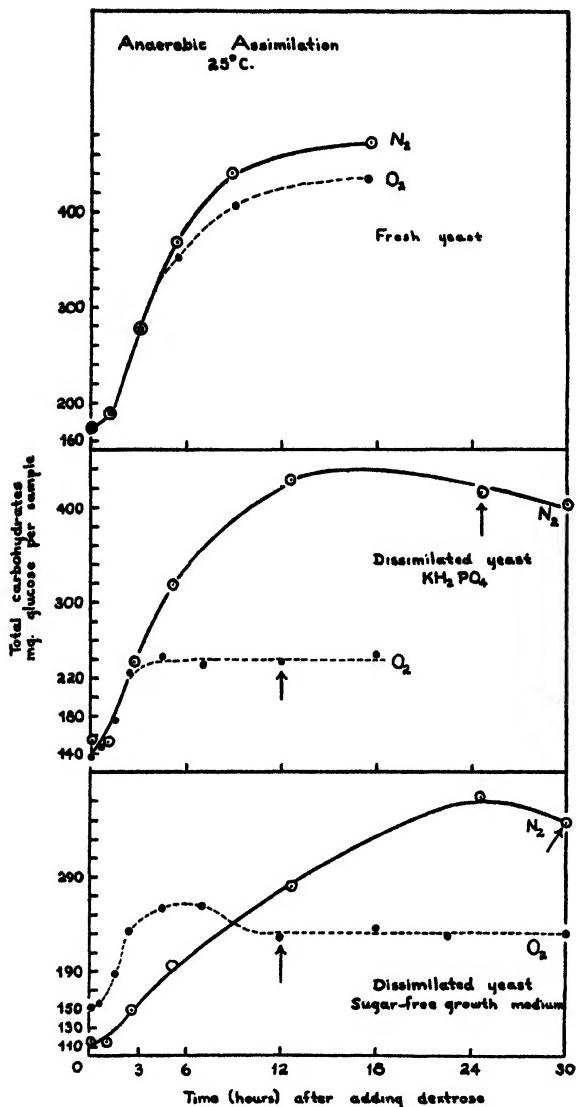
Fig. 2. Time-course of anaerobic carbohydrate assimilation. Data for aerobic assimilation (smaller symbols and dotted lines) are included for comparative purposes. Arrows mark points where all the dextrose had disappeared from the suspension. (Data of Stier and Simonds.)

by bubbling oxygen-free nitrogen through the suspensions during the entire course of the reaction.

Note the greater amount of total carbohydrate stored in the absence of air by all three types of yeast employed. Oxygen exerts an inhibiting effect upon carbohydrate assimilation when the final yields are compared. However, during the first 2 to 3 hours, no inhibition of assimilation in the presence of oxygen is seen in "fresh yeast" (and possibly in the yeast which was dissimilated in KH_2PO_4). The similarity of the course of

carbohydrate assimilation of "fresh yeast" in both O_2 and N_2 (Fig. 2) is probably significant since the yeast employed for these two experiments was obtained from the same lot and the experiments were done on the same day. In the curves marked "dissimilated yeast" the cells for the two N_2 curves came from one lot of GM yeast and the yeast for the two O_2 curves from another shipment of yeast. Therefore the data should not be rigorously compared as to the effect of O_2 upon the initial course of the reaction in each type of dissimilated yeast. It is apparent that a more careful scrutiny of the behavior of the initial portion of the reaction should be made in the presence and absence of oxygen. The information obtained may throw light on the way in which oxygen acts to limit the building-up of a maximum amount of carbohydrate.

IV

In the Meyerhof cycle theory of aerobic resynthesis it is proposed that polysaccharide formation results from the aerobic resynthesis of fermentation cleavage products (cf. especially, Meyerhof, 1925; and Burk, 1937, for other references). The following data obtained from dissimilated GM yeast are presented as a test of this theory as it applies to bakers' yeast.

We have recently begun the development of a perfusion method which will pass 500 or more ml. of solution per hour through the yeast suspension in the reaction chamber. Alcohol and other by-products of dextrose metabolism are thus kept at the lowest possible concentration during assimilation. In our first set of experiments with this method we obtained the following results: At the end of 14 hours of perfusing 5 per cent dextrose in $M/15$ KH_2PO_4 at a rate of 660 ml. per hour (aerobic conditions; 25° C.) the total carbohydrate content was 336 mg. of glucose per
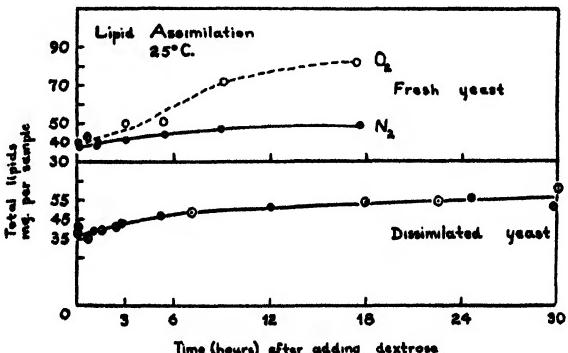

Fig. 3. Anaerobic lipid assimilation. In the upper part of the figure data for aerobic lipid assimilation (smaller symbols and dotted lines) are included for comparative purposes). ● assimilation in N_2. ○ assimilation in O_2.

unit number of cells. Cells from the same lot of yeast when assimilated in the usual manner (*cf.* section III) had a carbohydrate content of 315 mg. per unit number of cells; a non-limiting concentration of dextrose was still present in the suspension at the end of 14 hours of assimilation. Thus, carbohydrate synthesis proceeded to about the same value even though the by-products of dextrose metabolism were continually washed out of the reaction chamber. When cells from the same lot of yeast used in the above two experiments were perfused with 5 per cent alcohol in $M/15$ KH_2PO_4 for 12 hours the total carbohydrate content fell from the initial value of 177 mg. to 159 mg. per unit number of cells. These experiments indicate that carbohydrate synthesis takes place to the same extent in the presence or absence of the by-products of dextrose metabolism. In the presence of only alcohol the carbohydrate content was not increased, but rather decreased by about 10 per cent. Further experiments of this type are in progress.

The data we have obtained, thus far, indicate that during dextrose metabolism carbohydrate synthesis proceeds concurrently with alcohol production and that the alcohol produced in the medium is apparently not utilized for carbohydrate synthesis.

In connection with the resynthesis suggested by the Meyerhof oxidation quotients, Burk (1937) suggested that something "other than higher carbohydrate resynthesis" may be involved. Fig. 1 and 3 show that the ether extractable material also increases in amount during dextrose metabolism, the rate of accumulation and the maximum reached being higher in air than in the absence of oxygen in the case of fresh yeast. The dissimilated yeasts, however, store about the same total amount of lipids under aerobic conditions as under anaerobic conditions and much less than the "fresh" cells. Here again, the role of riboflavin in lipid synthesis should be carefully investigated.

Conclusions regarding the substances from which lipids are synthesized during dextrose metabolism cannot be given from the data at hand. Smedley-MacLean pointed out, in 1936, that sugars have been found to be the most efficient substances leading to storage of fat by yeast. Other compounds which give rise to increased fatty acid production in yeast were ethyl alcohol and sodium acetate. In all cases, aeration of the media was an essential condition for fat storage. MacLean proposed that the pathway to fat formation is *via* carbohydrate stored within the cell. Smythe (1938) has shown that bakers' yeast forms 2 to 4 times as much fat from pyruvic acid under aerobic conditions as under anaerobic

conditions. Whether the lipids are synthesized directly from the alcohol and/or other by-products which are produced into the medium, or from the polysaccharide or hexose within the cell remains for future investigations. We have recently started an analysis of this complex tangle by aid of our perfusion technique.

Summary

(1) In the presence of oxygen the maximum amount of carbohydrate stored in "fresh" yeast is about twice that stored in dissimilated (starved) cells which were shown to be deficient in riboflavin. The rate of carbohydrate assimilation for the first 2 to 3 hours is, however, almost the same in both types of yeast. After 2-3 hours carbohydrate ceases to accumulate in the dissimilated cells. By varying the flavin content of the starved cells it is hoped to find an explanation for the rather abrupt cessation of carbohydrate storage observed in dissimilated yeast cells.

(2) In the absence of oxygen the maximum amount of carbohydrate stored in fresh yeast and in dissimilated yeast is roughly the same: "fresh" yeast, 0.67 mg. glucose per million cells; KH_2PO_4-dissimilated yeast, 0.59 mg. per million cells; and yeast dissimilated in sugar-free growth medium, 0.74 mg. per millions cells.

(3) From the data available it appears that cells made deficient in flavin (and possibly other metabolic units) are only able to carry carbohydrate assimilation to a limited level in the presence of oxygen, whereas in its absence the potentialities of the carbohydrate assimilation mechanism are apparently unimpeded.

(4) In all the experiments at hand, oxygen exerts an apparent "inhibitory" effect upon carbohydrate assimilation when the final yields in oxygen are compared with those obtained under anaerobic conditions. However, the inhibitory effect of oxygen observed in our lots of "fresh" yeast may not turn out to be the characteristic behavior of bakers' yeast cells taken directly from their culture medium at their maximum rate of growth and then quickly prepared for assimilation experiments without loss of essential intracellular materials.

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DISCUSSION

Dr. Cori: What per cent of the total carbohydrate of the yeast was glycogen and what method was used for the separation of glycogen from yeast gum and hemicelluloses?

Dr. Stier: About half of the total carbohydrate was glycogen. The method used for separating the glycogen was that of Mayer (1923). The washed cells were treated with 60 p.c. KOH, the polysaccharides precipitated with alcohol, the yeast gum and glycogen dissolved in boiling water and the glycogen precipitated with saturated ammonium sulfate.

Dr. Cori: How did you determine it?

Dr. Stier: The glycogen which had been isolated according to Mayer's method was hydrolyzed with acid and the resulting reducing sugar determined, in these particular experiments, by a modification of Bertrand's method.

Dr. Stern: I understand that your experiments on the oxidative synthesis were done at 5° C. That brings up the question of the temperature coefficient of the Pasteur reaction. Kubowitz has done some experiments on the Pasteur effect in frog retina. Up to 35° C. the Pasteur effect as well as the respiration and glycolysis show a temperature coefficient which one would expect from chemical reactions. There occurred a sudden rise of the aerobic glycolysis at 40° C. and the Pasteur effect was completely abolished. The interesting point is that the critical increment thus observed is of the order of that encountered in the denaturation of proteins, which supports the view that the Pasteur agent is built according to the hemoglobin model. Kubowitz's experiments demonstrate that one may produce any kind of Pasteur effect by working at different temperatures.

Dr. Stier: We have studied assimilation only at 25° C. and 5° C. At these widely separated temperatures, which are presumably in the "normal" temperature range, carbohydrate assimilation in dissimilated yeast always exhibited the same characteristics. These were (*1*) greater storage of carbohydrate under anaerobic conditions, the carbohydrate content increasing continuously from the moment dextrose was added; (*2*) a relatively small initial increase in carbohydrate under aerobic conditions followed by a second phase where no further increase in carbohydrate occurred, even though a non-limiting supply of dextrose was present. The time to reach the beginning of the second phase was less at 25° C. than at 5° C.; the temperature coefficient (Q_{10}) was approximately 6. Possibly above some upper critical temperature, a different type of assimilation behaviour would be exhibited, just as in Kubowitz's experiments where the Pasteur effect was abolished at temperatures above the normal temperature range.

Dr. Gemmill: How does the yeast use its fat stores? Can they be converted into carbohydrate?

Dr. Stier: The problem of how the yeast cell uses its carbohydrate and fat stores has not as yet been systematically investigated using chemical methods. Stannard and I have made a beginning with manometric methods. Cells from young cultures showed a declining rate of respiration which was first-order with respect to time; the R.Q. remained 1.0 through ten hours of dissimilation, indicating utilization of carbohydrate reserves; anaerobic CO_2 production was found to be negligible, production of alcohol could not be detected. It was concluded that dissimilation of the carbohydrate stores was a purely respiratory process.

Our recent chemical analyses of the contents of commercial bakers' yeast after three days dissimilation indicate that the carbohydrate and fat content of the cells decrease. We have not yet determined the time-course of carbohydrate and fat utilization during this longer interval of starvation. Future studies of this type should show when in the course of dissimilation carbohydrate and fat stores are utilized and should give an indication of whether the stored fat is first converted to carbohydrate before it is utilized in endogenous metabolism.

Dr. Burk: I had the opportunity to see some of these data a month ago in the thesis of Herbert Sprince, working with Stier. I think it is interesting and necessary to consider some of the absolute quantities involved here as well as the relative ones.

A point which I would like to mention especially is the rather small proportion of all the quantities we have been considering this morning in relation to the total glucose consumed. The situation here is quite different from that which we are used to considering in the synthesis of muscle or liver. To give some idea of the figures (I am speaking now of the dissimilation of yeast under anaerobic conditions, which is perhaps the most interesting case), at the end of the experiment of 140 hours, there were for 160 units of glucose consumed, 52 units of alcohol formed, and the total carbohydrate in the yeast was only 7 units, of which the glycogen was 4 units; the total dry weight was 8 units, and the fat, incidentally, was zero. This, I think, has quite a bearing on the finding of more "synthesis" (and glycogen) anaerobically and aerobically, in contrast to the almost universal finding by the other workers a good deal more synthesis aerobically than anaerobically. Stier thinks that quite possibly the flavin content and "dissimilated" state of his yeast plays a marked role in this connection.

Another thing which I noted was that the balance sheet of glucose consumed on the one hand and products yielded on the other was about 30 per cent unaccounted for; that is to say, the alcohol formed plus the equivalent amount of carbon dioxide, plus the dry weight, makes up, both anaerobically and aerobically, the 30 to 40 per cent discrepancy, less than the glucose consumed. It is possible that quite a bit of gum was formed which was not included in the analysis, also some succinic acid and glycerol; aerobically the discrepancy might be largely accounted for by the oxygen consumption and CO_2 production, which were not measured. On the other hand, anaerobically we certainly have this discrepancy.

Dr. Stier: Several discrepancies are inherent in the data which you have just quoted. It was not feasible, at that time, to trap the alcohol which

escaped with the gases which were bubbled through the suspension. Also, since the samples were fixed in alcohol some of the cellular material was lost. Obviously, the apparatus and procedures will have to be modified before we can start work on the glucose balance sheet. We plan to employ micro-carbon methods. A more accurate estimate of the fermentation cleavage products in the non-cellular fraction should thus be obtained. Loss of cellular material in the fixative could be obviated by use of the cooling method recently employed by us.

Dr. Burk: Essentially all of the discrepancy should be in the non-cellular fraction because the cellular fraction itself is relatively very small, almost negligible so far as the balance sheet is concerned, and the loss due to extraction of this dry weight would be totally negligible here.

Dr. Stier: The loss in the alcohol fixative was approximately 5-10 p.c. of the dry weight.

Dr. Bernheim: Is it not true that this yeast has been trained to do a definite job? In other words, to produce CO_2 rapidly? If I understand Burk's figures, it is inefficient in storing energy for itself. Wouldn't the picture be quite different if you used wild yeast?

Dr. Stier: A wild yeast would probably give a different relation between the glucose consumed and glucose stored. We are planning to study assimilation in various other strains of yeast as soon as we can simplify the procedure of culturing the large quantities of yeast required for investigations of this type. We have, thus far, used only commercial bakers' yeast because of the convenience of obtaining it in bulk.

Dr. Burk: I think these experiments are interesting and unique in several ways. They represent one extreme, the extreme where one would expect the least efficient synthesis. The yeast has been reduced to almost its lowest powers; the studies are comparable to those on what happens when food is given to dog or man starved one or several weeks.

Another interesting point is that these experiments represent the simultaneous measurement of anabolism and catabolism; whereas, in the past one has had mostly simultaneous measurement of aerobic and anaerobic processes, in this case it is simultaneous anabolism and catabolism which are measured also.

Another thing to bear in mind in these experiments in relation to the Pasteur effect, is that here the main starting point has been *glucose*, whereas in past "Meyerhof-Pasteur" experiments in regard to resynthesis the starting point has concerned *fermentation products* of glucose. These experiments thus represent a somewhat different and more modern treatment of the problem.

Dr. Stannard: You mentioned changes in the

rate of oxygen consumption during assimilation. I wonder if you would care to tell us some of your ideas on the correlation between the changes in oxygen consumption and the steps in assimilation.

Dr. Stier: In the experiments which I mentioned (see Stier and Newton, 1939) the rate of oxygen consumption was constant during about the first two hours, then steadily declined, finally becoming constant after about the third hour. The most rapid increase in carbohydrate content of the yeast cells occurred during the first constant-rate phase of oxygen consumption. It was found that the lowered rate of respiration, after the third hour, was not brought about by the decline in dextrose concentration or by the change in composition of the air in the respirometer vessels. Removal of the accumulated by-products of dextrose metabolism during the second constant-rate phase, by washing and resuspending the cells in fresh dextrose-phosphate solution, increased their rate of respiration by only 30 p.c. When fresh (unassimilated) cells were suspended in fermented dextrose-phosphate solution, obtained during the second constant-rate phase, they exhibited initial rates of respiration which were identical with the controls.

The working hypothesis selected for future work on this problem views the change in rate of respiration during dextrose metabolism as resulting directly as a consequence of changes occurring during assimilation in the finer cytological structure of the cell, *i.e.,* in the "cytoskeleton, following Peters and Needham, or indirectly through the action of some by-product of dextrose metabolism after the cellular structure has become sufficiently altered during the course of assimilation. It is planned to continue investigations of this problem by aid of a perfusion technique which we are developing for use within respirometer vessels.

Dr. Stannard: Since the addition of dextrose at the end of the experiment did not bring the lowered rate of oxygen consumption back to the original rate, it would seem that there must be some change other than that in the available substrate. Possibly the change reflects the changes in active enzyme content, a loss in this case.

Dr. Stier: It is possible that such a change might also be involved in bringing about the observed reduction in rate of respiration. This point should be considered in future investigations.

THE MECHANISMS INVOLVED IN THE TRANSFER OF OXYGEN IN FROG MUSCLE

J. Newell Stannard

In spite of the large variety of experiments available which involve measurements of the oxygen consumption of frog muscle, relatively little attention has been paid to the oxygen transfer mechanisms involved. Such relative neglect is entirely understandable. As long as the concept of a common initial pathway for the aerobic and anaerobic decomposition of carbohydrate was in favor, the chemical steps involved in the oxidation of the product or products of glycolysis seemed so far removed from the possible scene of liberation of the contractile energy as to be devoid of any compelling interest. In addition, the presence of ample quantities of cytochrome and its oxidase (Keilin, 1925) and the fact that the oxygen consumption could be almost completely inhibited by cyanide (Meyerhof, 1919, 1930; Ellinger, 1924) left little doubt in the light of modern work that the oxygen transfer in frog muscle was mediated by the standard Warburg-Keilin (cytochrome-cytochrome oxidase) system.

However, recent work on muscle has not served to strengthen the already overemphasized evidence that aerobic and anaerobic metabolism do necessarily traverse common initial paths to the extent proposed originally. A review of the literature reveals conflicting points of view and contradictory evidence. Also the problem of the mechanism of oxygen transfer assumes greater importance because of recent allegations that oxidative processes may contribute directly to the energy liberation in aerobically working muscles (Sacks, 1938).

The present paper aims to present the results of experiments designed to study the oxygen transfer mechanisms in intact frog muscle using specific chemical inhibitors. It is assumed that the behavior of a given enzyme or carrier toward inhibitors will be roughly the same whether this enzyme or carrier is in a relatively pure state in the test tube or functioning *in vivo*. The presence and relative activity of, for example, cytochrome oxidase can, with reservations, be studied in intact tissues by this method. In addition behavior toward inhibitors not expected from test tube preparations may be indicative of new factors in the operation of known systems imposed by the fact that these systems are contained in intact cells or of new components not yet isolated *in vitro*. The limitations of this technique are fully realized and the limitations of work with intact tissues are strict (*cf.* Green, 1938). Yet it is felt that a useful purpose can be served by applying this method.

Separation of the resting and activity oxygen consumptions

It has long been a problem of interest to know whether or not the increased rate of respiration of active muscle represented simply an increased rate of activity of mechanisms "idling" in the resting muscle or the addition of qualitatively new components. Differences in the metabolism of many tissues and organisms at different levels of metabolic activity have been found in many instances (*e.g.* salivary gland by Deutsch and Raper, 1938; grasshopper embryos by Bodine and co-workers, 1934; sea urchin eggs by Runnström, 1928; *Neurospora tetrasperma* by Goddard, 1935-36, Goddard and Smith, 1938; carrot roots and leaves by Marsh, 1939; wheat with and without infection by powdery mildew, Allen and Goddard, 1938; anaerobic processes in resting and active frog muscle, Cattell and Feit, 1937). Although some of these cases may not require the postulate of qualitative differences in a final analysis, there is no reason for assuming that the oxygen transfer of all aerobic cells utilizes a single system (note especially discussion by Keilin and Hartree, 1939). In the case of frog muscle the fact mentioned earlier, that the total respiration could be inhibited by cyanide, seemed to settle the question in favor of the presence of but a single oxygen transfer system. It was, therefore, surprising in an attempt to substitute sodium azide for cyanide as a respiratory inhibitor to find that the oxygen consumption of resting muscle was relatively insensitive to this poison (Stannard, 1939). Keilin had shown (1936) that sodium azide was a potent inhibitor of yeast respiration (at acid pH), and its effect on the cytochrome-cytochrome oxidase system has received careful study (Keilin and Hartree, 1939). Since Keilin (1936) had found the inhibitory action on yeast respiration to be markedly dependent on pH and since the pH of the Ringer's solution (7.4) was in the range in which no effect was observable in yeast, it seemed probable that the lack of effect on resting muscle was due to this phenomenon. Armstrong and Fisher (1939) find a similar effect of pH on the inhibitory action of NaN_3 on the frequency of embryo salmon or *Fundulus* hearts and can account for it adequately on the assumption that only the free acid (HN_3) is effective in producing the inhibition. However, as shown by Stannard (1939), reducing the pH of the Ringer's to as low as 4.6 has little effect on the sensitivity of the resting oxygen consumption of muscle. Furthermore, as will appear presently, there is ample

evidence for penetration and effectiveness of this inhibitor even at pH 7.4.

In marked contrast to its lack of effect on the resting muscle any increments in respiration caused by stimulation of the muscle either electrically or chemically were markedly sensitive to azide. This was true even at pH 7.4. Chemical contracture induced by acetylcholine or KCl caused no rise in oxygen consumption in the presence of 0.002 M azide. The mechanical shortening was not hindered in any way. Of perhaps greater interest is the fact that the increased oxygen consumption following electrical stimulation was likewise prevented.

Saslow (1937) has studied the large increases in oxygen consumption of frog muscle treated with subcontracture concentrations of caffeine. It was considered of interest to determine the effect of sodium azide on this increased oxygen consumption, especially since Saslow found his data consistent with the idea that caffeine liberates continuously the processes normally set off discontinuously by stimulation. (cf also Hartree and Hill, 1924; Meyerhof, 1921). For technical reasons the effect of the inhibitor could be better studied when the elevated rate of oxygen consumption was constant with time. The results were a grati-

fying confirmation of those obtained by other means of stimulation. Concentrations of azide from 10^{-5} to 10^{-3} M caused progressively greater inhibition of the extra oxygen consumption which remained at the new level for each concentration. Below 10^{-5} M there was no effect at all. Above 10^{-3} M very little further inhibition took place and it soon became apparent that the level of maximum inhibition coincided precisely with the normal rates of respiration of resting muscle.

The data were so regular in this range that a simple formulation based upon the mass law could be applied and when so applied appeared valid. This is shown in Fig. 1 as a logarithmic plotting of the ratio of the fraction of the respiration inhibited $(1-N)$ to that uninhibited (N) against the concentration of azide. A sharp discontinuity appears at approximately 10^{-3} M azide when the raw data are used, as might be predicted from the fact that no further inhibition takes place above 10^{-3} M. If the data are "corrected" by substracting the resting respiration on the assumption that this persists unchanged in the presence of the caffeine a linear relation appears without any discontinuity. The slope of the line is equal to unity indicating a one to one relation between inhibitor and active enzyme groups combined. Whether or not this method of formulation is the best to apply, the discontinuity at 10^{-3} M (uncorrected data) is equally apparent if one plots either the per cent respiration remaining (cf. Fig. 2) or the absolute rates against the concentration of azide.
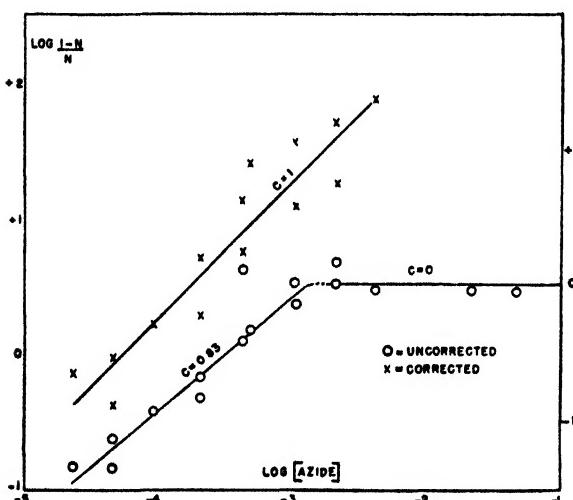
Fig. 1. Data for the effect of azide on caffeinized muscle plotted according to a simple mass law formulation. It is assumed that azide combines with enzyme as expected from $[E] + C [Az] = [E Az_o]$, that $[E]$ can be represented by the fraction of the respiration uninhibited (N) and $[E Az]$ by the fraction inhibited $(1 - N)$ by azide. Thus a logarithmic plotting of $(1 - N)/N$ against the concentration of azide $[Az]$ should yield a linear relation if the law holds. The slope of the line represents the constant C above. The lower line shows the data when plotted directly: the upper line when "corrected" by subtracting the resting respiration. This is a mass plotting including many experiments. Right hand scale for upper line. (cf. Stannard, 1939, for further details).
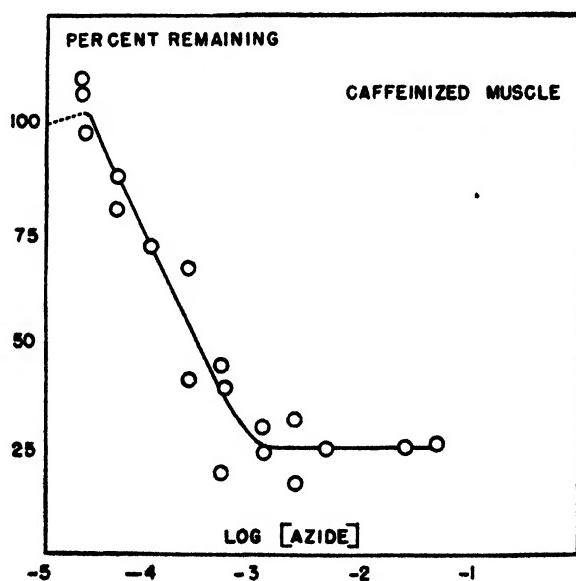
Fig. 2. The per cent respiration remaining in a series of experiments with caffeinized muscle plotted against the concentration of azide (logarithmic scale for concentration to conserve space). Note the discontinuity at 10^{-3} M azide.

Since azide inhibits the oxidation of *p*-phenylene diamine by muscle but not the increments in O_2 consumption due to the addition of methylene blue or pyocyanin (Stannard, 1939) it seems logical to suppose that its action is upon the cytochrome oxidase, or in the terminology of Keilin and Hartree (1939) the cytochrome "a_3".

A serious objection to such a qualitative separation of the systems of oxygen transfer in resting as compared with active muscle might be found in ideas based upon the concept of "unsaturation" of the enzyme surfaces with substrate. This view, first expressed by Warburg (1937), states that at less than the maximum respiratory rate the oxidizing enzymes may be "unsaturated" with substrate and consequently considerable portions of the enzyme may combine with poison without noticeable effect on the rate of respiration. Recently Commoner (1939) has emphasized the dependence on the concentration of substrate present of the action of cyanide on the respiration of yeast. Thus the lack of sensitivity of resting muscle to azide might be due only to lack of available substrate. Whether or not the theoretical interpretations placed upon this "saturation" phenomenon by various authors are correct (see especially Fisher and Cameron, 1938) studies of the lactate production in frog muscle by measurements of the anaerobic glycolysis (*cf.* Stannard, 1938a, 1939) show that there is no lack of available substrate under conditions where sodium azide causes no inhibition of the resting respiration. In fact the presence of the discontinuity at 10^{-3} M azide even in the presence of the high rates of oxygen consumption in caffeinized muscle should be sufficient evidence that "unsaturation" cannot account for the results obtained.

It should be mentioned that the effects of this inhibitor are completely reversible if the muscle is not left in contact with the poison for more than thirty to forty minutes (at 0.002 M). With longer periods of exposure the muscles proceed into rigor in spite of removal of the poison. Another point which deserves mention is the fact that azide causes a slight contracture. This was determined by means of kymographic records of the shortening and in addition the expected increases in lactate production were found (*cf.* Stannard, 1939). This contracture results in an increased rate of oxygen consumption when produced by the addition of low concentrations of azide to resting muscle. At higher concentrations (0.005 M and above), although the contracture is more marked, no increase in oxygen consumption follows since the oxidation of the lactate being produced (and control experiments showed that it actually was appearing in ample quantities) is prevented by azide. The presence of a contracture and the eventual loss of irritability complicates the analysis in some respects, but does not vitiate the results. Experiments now in progress indicate that hydroxylamine in proper concentrations may effect a separation of these phases of metabolism just as clearly as azide, but without inducing a contracture or an eventual loss of the excitability.

On the nature of the resting respiration

It might be concluded from the fact that the respiration of the resting muscle remains unchanged even in sufficient concentrations of azide to completely prevent any increments in oxygen comsumption due to stimulation, that the connection between the resting respiration and those processes underlying oxidative recovery is remote. In addition it might seem that the energy produced in the resting muscle has little connection with the maintenance of excitability. The first postulate is difficult to avoid. The second need not necessarily be true. Thus the azide might not only inhibit the cytochrome oxidase but also prevent the utilization of the energy produced in respiration. In support of this it might be said that with hydroxylamine the excess respiration can be prevented without the muscles becoming inexcitable. Furthermore even with azide the effect is not simultaneous on both respiration and excitability (*cf.* Stannard, 1939).

If the action of azide is on the cytochrome oxidase then the resting respiration must not traverse this system. Yet, as indicated earlier, the resting O_2 consumption is inhibited by cyanide. There is really no paradox here, however, for it has been shown (Stannard, 1939) that although cyanide inhibits both resting and excess O_2 consumption it does so with sufficiently different kinetics to indicate that the cyanide is probably combining with different enzymes or in different loci. The nature of this cyanide-sensitive azide-insensitive system is quite unknown. However, it is not without precedent for these two compounds to behave differently *in vivo*. For instance, a rather specific effect of azide is seen in its action on certain bacteria where the synthetic processes associated with respiration can be prevented and yet complete oxidation of substrate can still be accomplished. In fact the oxidation is complete only in the presence of the inhibitor (*cf.* Clifton and Logan, 1939; Geisberger, 1936; Stephenson, 1939, p. 38). Of particular interest are the as yet unpublished findings of Van Dock (work done at the Marine Biological Laboratory, Woods Hole, Mass. in 1938 and communicated personally by K. C. Fisher) which showed that *Astasia* in the absence of external glucose presents a respiration which is sensitive to cyanide but not at all sensitive to azide. In the presence of glucose the respiration is poisoned by both inhibitors.

The system of oxygen transfer operative in resting muscle is not necessarily markedly different from better known ones (*cf.* Keilin and Hartree, 1939). On the other hand the existence of cells devoid of cytochrome, yet whose respiration is sensitive to cyanide, together with the discovery of copper-containing enzymes which are cyanide-sensitive (*e.g.* polyphenol oxidase, Kubowitz, 1937, Keilin and Mann, 1938; laccase, Keilin and Mann, 1939) indicates that there may still be room in the systems of oxygen transfer in animal tissues for new components. The cyanide-sensitivity of the resting respiration precludes the possibility that it represents an independent functioning of the yellow enzyme system. Likewise the sensitivity to cyanide excludes cytochrome-b (*cf.* Stotz, Sidwell, and Hogness, 1938). Experiments to date (three) indicate that the R.Q. of resting muscle in 0.002 M azide is very close to unity (average 1.03) thus making it improbable that the resting respiration represents the metabolism of fat (*cf.* Kuhn and Meyer, 1929).

The effect of carbon monoxide

A commentary on the relative scarcity of experiments dealing with the oxygen transfer mechanisms in intact muscle is found in the fact that apparently carbon monoxide had never been used until the work of Fenn and Cobb (1932a and b). It was, therefore, with some alarm that these authors found that carbon monoxide stimulated rather than inhibited the oxygen consumption of frog muscle. The stimulation was of the order of 100 per cent of the resting rate. The experiments were controlled in every way possible, and it was shown later (1932b) that the stimulation represented oxidation of the CO to CO_2. This property was found to be virtually limited to skeletal and cardiac muscle. The stimulation itself was confirmed by Schmitt and Scott (1934). However, except for the paper by Carleton and Fenn (1938) mentioned below, the essential facts or mechanism of this effect have received little further attention, although significant studies of the effect of CO on mammalian tissues have appeared (*cf.* Laser, 1937).

In the study of the oxygen transfer mechanisms being summarized here it was, of course, necessary to know whether this peculiar property of skeletal and cardiac muscle represented some unique variation in the functioning of the cytochrome-cytochrome oxidase system or an independent system. Since CO inhibited the indophenol oxidase of sheep's heart muscle (Keilin, 1927) Fenn and Cobb reasoned that the CO burning must be in reality superimposed upon an inhibition due to CO. However, no direct evidence was available.

Later Carleton and Fenn (1938) showed that the excess metabolism due to CO was little if any modified by the large increase in metabolism caused by treatment with KCl in concentrations above 0.02 molar. The same was true of the increased metabolism and contracture caused by acid or alkali within certain limits. The present work has provided a fairly adequate set of data upon this subject and these will be presented in some detail since, unlike the previous section, none of the material has been published hitherto.

The essential observations may be grouped under two questions. (1) Will the amount of CO burned be dependent upon the rates of respiration prevailing or be otherwise related to the normal respiration? (2) Can the CO effect be inhibited selectively? The first question arose in part as a result of the work of Hursh (1935). Hursh found less recovery oxygen or recovery heat in $CO-O_2$ mixtures than in air, and interpreted the results provisionally as indicating some change in the efficiency of recovery. However, as Hursh realized, an alternative explanation would be that there was less excess metabolism due to CO at a time when the metabolism was high due to activity. The data of Carleton and Fenn would tend to negate this hypothesis, although these workers obtained some indication of a decrease in CO burning at the peak of the KCl effect.

Table I presents a summary of experiments in which the rates of respiration were measured before and after addition of substances used to raise the metabolism, and again after addition of CO. Controls, not shown in the table, included measurements of the increment due to CO in untreated muscles. This averages 50-55 mm^3./gm./hr. Superficially it appears that the excess metabolism in CO persists in spite of increases in the basal rate of respiration by lactate, pyocyanin and KCl, but not in the other cases. At first glance it might seem that this represented only a "ceiling effect" *viz.* the rate of respiration was at a maximum for the prevailing O_2 tension, etc. and no further increase could be expected. In fact in an earlier experiment with 2.3 p.c. lactate (not included in the table) when the basal rate was 136 mm^3./gm./hr. it increased to only 139 in a $CO-O_2$ mixture. However, this cannot be the explanation in every case since, for example, with isotonic dextrose the rates are actually much lower than with KCl yet no increase due to CO was apparent. The muscles were of approximately the same thickness in all experiments. Furthermore the rate in dextrose could be doubled by addition of methylene blue without changing the O_2 tension, and changes in O_2 tension did not

TABLE I

The CO excess in 80 p.c. CO/20 p.c. O_2 mixtures after addition of substances which increase the basal rate of oxygen consumption.

Substance	Concentration	No. of experiments	Before addition	After addition	With CO	Excess
				mm²./gm./hr.		
Lactate	0.2 p.c.; 2 p.c.	4	41.5	76	109	+33
Pyocyanin	0.005 p.c.	1	41	92	119	+27
KCl*	0.0015 - 0.116M*	21*	40*	123*	166*	+43*
Dextrose	4.5 p.c.	6**	40	77	75	−2
Dextrose + NaCl	4.5 p.c.	4	40	39	63	+24
Methylene blue	0.001 M	3	29	154	153	−1
p-phenylene diamine	0.001 M	4	22	66	65	−1
Nadi reagent	0.001 M	2	44	78	70	0
Caffeine	0.04 p.c	5	37	139	143	+4

* Data of Carleton and Fenn (1938)

** One experiment with isotonic sucrose, but no difference in results

increase the rate in dextrose alone. Yet no change in the effect of CO was apparent.[1]

In any event this was not the complete story. A striking indication that these experiments did not give a true picture of the effect of CO was found with the Nadi reagent. It will be observed in Table I that in the presence of Nadi reagent no increase over the rates with Nadi reagent alone took place in CO—O_2 mixtures. If the cytochrome oxidase is at all functional in frog muscle, why should not oxidation of the Nadi reagent be inhibited by CO? As shown in Fig. 3 even raising the CO content of the mixtures to 95 per cent resulted in no evidence of inhibition of the oxygen consumption when compared with nitrogen-oxygen mixtures of the same oxygen content. In fact a slight increase was evident in CO. But a test-tube experiment showed conclusively that the production of indophenol blue (estimated colorimetrically) was definitely inhibited in these same muscles by 80 p.c. CO—20 p.c. O_2 mixtures. Furthermore the inhibition was markedly sensitive to light. The only logical explanation for these facts is that CO inhibits that fraction of the oxy-
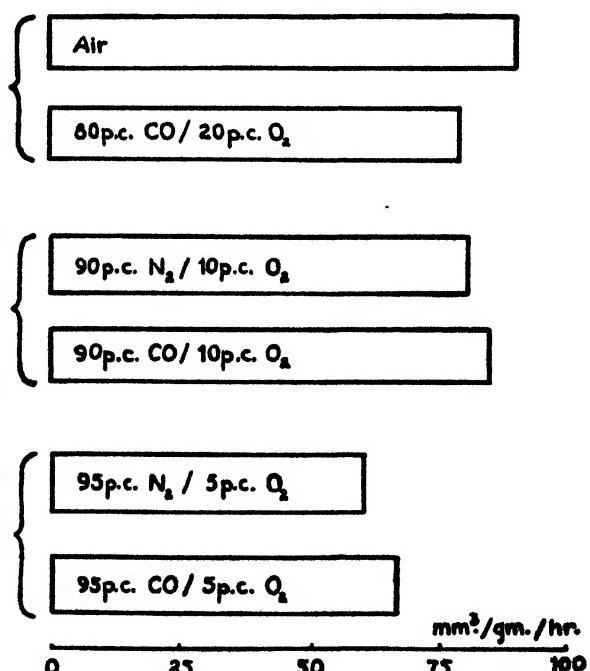
[1] It will be noted that the addition of sodium chloride to muscles in isotonic dextrose brings the rate of oxygen consumption back to the resting level. This has been observed by Fenn (1931). The striking fact here is that with the return to resting levels the increment due to CO reappears. The final concentration of NaCl was 0.05 per cent.

Fig. 3. Illustration of the effect of various CO—O_2 and N_2—O_2 mixtures on the oxygen consumption of muscle in the presence of Nadi reagent.

gen transfer mediated by the cytochrome-cytochrome oxidase system (exclusive possibly of the autoxidizable cytochrome-b) but is burned in equivalent amounts up to a limit set by some undetermined factor or factors. The resultant represents a rate of respiration nearly identical with that in the absence of CO.

To what extent this conclusion applies to each of the other means of increasing the metabolism in which no excess is apparent by ordinary measurement is not certain. However, a series of experiments with caffeinized muscle afforded striking confirmation. For these determinations the amount of oxygen consumed was compared with the total amount of gas used. A modification of the technique of Fenn and Cobb (1932b) was employed. The total gas used was measured by means of a special respirometer which permitted the index drop to be reset without opening the chamber to the air. This was done by introducing mercury to diminish the volume of the gas space. The mercury did not come in contact with the muscles. After a long enough experimental period to insure an easily measurable change in composition of the contained gases the whole apparatus could be filled with mercury and the gas displaced into a mercury-filled sampling tube for analysis on the Haldane gas analyzer. The oxygen consumption was measured by analysis for oxygen and the CO disappearing estimated by the difference between this and the total gas used.

The following expression was used in the calculations:

$$O_2 \text{ used} = V(x\text{-}z) + z\Delta V$$
$$CO \text{ used} = \Delta V - [V(x\text{-}z) + z\Delta V]$$

where V = initial volume of gas, x = initial per cent O_2, z = final per cent O_2, ΔV = diminution in volume over the experimental period.

The data are presented in Table II. Particular attention is directed to the last two columns. Here it is seen that in resting muscle the CO used amounts on the average to 89 per cent of the O_2 used. If the amount of CO used is multiplied by 1.5 to account for the half mol of O_2 used per mol of CO and the rate per gram of muscle per hour calculated, a "calculated excess" is obtained (last column) which agrees very well with the average excess metabolism measured in ordinary respirometers. In the case of caffeinized muscle as shown in detail in Table III and in summary in Table I, experiments in which $CO-O_2$ mixtures are added to muscles in 0.04 p.c. caffeine in Ringer's solution in ordinary respirometers indicate very little excess in CO. Note the effect of light in the third experiment. Yet there is actually almost precisely the same amount of CO burning in caffeinized as in resting muscle. This is shown by the gas analysis technique in Table II. Although the amount of CO used is a much smaller percentage of the total gas used (average

Table II

The amount of CO burned in resting and caffeinized muscle.

Control	ΔV	x	z	O_2 used	CO used	CO/O_2	Calc. excess
mm³./gm./hr.	cc.	p.c.	p.c.	cc.	cc.	p.c.	mm³./gm./hr.
			Resting muscle				
36	0.755	20.40	19.70	0.381	0.374	98	78
65*	0.616	16.25	15.50	0.342	0.274	80	44
79*	0.580	16.56	15.53	0.419	0.161	38	33
27	0.643	19.41	18.96	0.263	0.410	156	57
—	0.972	18.08	16.83	0.558	0.414	74	48
					Average	89	52
			Caffeinized muscle				
148	0.975	23.13	21.14	0.814	0.161	20	42
143*	0.925	26.16	24.70	0.690	0.235	34	73
154	0.790	22.04	20.40	0.686	0.104	15	44
138	0.666	23.97	22.71	0.554	0.112	20	42
					Average	22	51.5

* Rate in CO/O_2 mixture; others in air.

TABLE III

The apparent effect of CO on caffeinized muscle.

O₂ content	Without CO	With CO	Excess	Difference
p.c.		mm.³/gm./hr.		p.c.
20	107	110	+3	+3
20	143	152	+9	+6
5	72*	80 (D)*	+8	+11
		87 (L)*	+15	+21
20	154	165	+11	+7
22	154	139	−15	−10
24	138	151	+13	+9
Average	139	143	+6	+7

* Not included in average

18 per cent of the total) the rate of CO burning (calculated excess) agrees remarkably well with the series on resting muscle. This can be interpreted only as an indication that the normal oxygen consumption of the caffeinized muscle is actually inhibited by the CO, but that CO is burned in equivalent amounts so that the apparent total oxygen consumption remains approximately the same. The type of evidence presented here goes far towards confirming the suspicion already voiced by Fenn and Cobb (1932a) and Schmitt and Scott (1934) that the CO burning is superimposed on an inhibition of the normal respiration.

The data in Table II require further comment. The experiments on resting muscle were done partly to prove the adequacy of the method. Since the burning of CO by tissues seemed rather improbable from a teleological standpoint the original observations of Fenn and Cobb have been received with some incredulity. (In fact it can be said now that the writer began work on this problem with the intent to disprove the notion that CO can be oxidized by these tissues.) However, every type of control experiment has served to prove the method. Thus cells which do not burn CO (e.g. yeast in the presence of external substrate) showed less than a 5 per cent difference between the total gas consumed and the oxygen consumed. Using muscles without CO the agreement was within 4.1 per cent. The criticisms of Haldane and Priestley (1935) definitely do not hold. The difference in solubility between CO and nitrogen either in the solutions in the respirometer or in the Haldane gas analyzer accounts for but a small fraction of the CO used. Also the Haldane analyses of the final mixture were made in duplicate and almost always after analyses of mixtures containing nearly the same amount of

CO. This eliminates combination of CO with the alkali in the Haldane analyzer as an explanation. The combination of CO with all of the intracellular hemin compounds, to say nothing of the cytochrome alone, would be complete in five minutes of an experiment in which the extra metabolism persists for five hours or more.

Experiments designed to find whether or not the CO burning could be inhibited specifically are presented in Tables IV and V. It will be recalled that Fenn and Cobb (1932a) found that both the normal and the excess respiration in CO were inhibited to about the same extent by KCN. Also no increase in lactate formation was necessary for the oxidation of CO, as shown by experiments with bromacetate. During the work with sodium azide the effect of this inhibitor on the CO excess was determined. As shown in Table IV concentrations of azide from 0.21 to 0.00004 M did not inhibit the resting respiration (compare "resting" column with "azide alone") but all concentrations of 0.00043 M and above prevent almost completely the excess respiration in CO. In the middle concentration ranges the slight contracture produced by azide causes a rise in the base line (cf. Stannard, 1939). If this increment in respiration brings into action the cytochrome-cytochrome oxidase system the apparently complete inhibition of the CO excess at 0.00043 M might really be a composite of inhibition of this increment and some superimposed CO burning. But at 0.005 M azide and above, the activity of the cytochrome system is completely inhibited by the azide. Hence the lack of CO burning must be interpreted as a true inhibition of this process as well.

More striking are the results with hydroxylamine shown in Table V. This inhibitor has little

TABLE IV

The effect of sodium azide on the excess metabolism in 80 p.c. CO. 20 p.c. O₂ mixtures.

Conc.	No. experiments	Resting	CO alone	Excess	Azide alone	With both	Excess*	Inhibition
(M)				mm.³/gm./hr.				p.c.
0.21	1	43	72	29	49	43	—6	108
0.01	1	30	—	—	28	37	+9	—
0.0072	1	22	64	42	30	23	—7	111
0.0043 to 0.0032	6	30	77	47	40	46	+6	92
0.0024 to 0.0019	7	29	66	37	49	54	+5	92
0.00043	1	30	74	44	68	67	—1	101
0.000043 to 0.00004	2	32	87	55	34	66	+32	42

* Calculated as above rate with azide alone

if any inhibitory effect on the resting respiration in the concentrations used and no uniformly stimulating effect. The inhibition of the CO excess is, on the average, complete. The control resting respiration in the experiment on 8/22 was abnormally high so that the normal CO excess for comparison with that in NH₂OH was low enough to produce apparently 142 per cent inhibition of the CO excess. If the average resting rate in a large number of experiments (30 mm.³/gm./hr.) is used the inhibition calculated becomes 96 per cent.

There were indications in the experiments with azide that the CO excess was more sensitive to this reagent than the increments in respiration caused by electrical or chemical stimulation (see above and Stannard, 1939). With hydroxylamine this was definitely true. Thus on 2/15, 0.0026 M

NH₂OH caused only 31 per cent inhibition of the excess metabolism due to caffeine in the first hour, and 38 per cent in the second hour, while as shown in the table the CO excess was 86 per cent inhibited. In another experiment on 2/14, thousandth molar NH₂OH caused only 39 per cent inhibition of the excess oxygen consumption in caffeinized muscle.

Concluding remarks

The facts outlined above may be taken as evidence that the oxygen transfer for the excess metabolism due to stimulation, the resting respiration, and the oxidation of CO is mediated by qualitatively separable systems. It also seems apparent that the increments in respiration caused by stimulation traverse the classical cytochromecytochrome oxidase system. The exact compon

TABLE V

The effect of hydroxylamine on CO excess.

Experiment	Conc.	Air	With CO	With NH₂OH	With both	Inhibition of CO excess
	(M)			mm.³/gm./hr.		p.c.
8/20	0.0021	30	81	29	40	80
8/22	0.0021	54	78	42	32	142
2/8	0.0043	21	91	37	43	92
2/10	0.0021	33	79	37	42	89
2/10	0.021	36	92	41	32	108
2/15	0.0026	22	73	36	43	86

* Excess calculated as above rate with NH₂OH alone.

ents of the other systems are not clearly established as yet.

A speculation on the nature of the system involved in the oxidation of CO might be permitted. The activity of catalase is known to be extremely sensitive to both azide and hydroxylamine (Keilin and Hartree, 1934; Blaschko, 1935a). In both cases the effects are obvious at much lower concentrations of inhibitor than usually necessary for inhibition of cell respiration. Blaschko (1935b) has shown that in some tissues there is no relation between inhibition of catalase by NH_2OH and inhibition of cell respiration. In other tissues there seemed to be some relation. The lack of parallelism between the effects of azide and hydroxylamine on the CO excess and on the excess due to caffeine might be interpreted as evidence that the CO oxidation depends upon the functioning of catalase. This would not be entirely unexpected in view of the coupled oxidations studied by Keilin and Hartree (1935-36) which depend upon the cooperative action of an oxidase and catalase. There are some objections to this explanation. Thus the crucial experimental evidence is not yet available, and there is no parallelism between the catalase content of tissues and their ability to burn CO. Surely, however, the present data indicate that the enzyme responsible for oxidation of CO is separable from both the resting and activity systems. Yet it may be very similar in properties to those already described. The demonstration by Negelein (1931) that CO can be burned to CO_2 by green and *"mischfarbene"* hemin in alkaline solutions at low O_2 tension shows that this enzyme in muscle may easily represent another variation of the ubiquitous hemin-containing respiratory enzymes. Obviously *in vivo* this system, whatever its nature may prove to be, must serve some other purpose than the oxidation of CO. Hence its further elucidation is a problem of importance.

The possible nature of the enzymes concerned with the resting respiration has been discussed in a separate section. It should be noted that Szent-Györgyi (1930) and Banga and Szent-Györgyi (1932) have separated the respiration of many tissues into fractions on the basis of sensitivity to arsenite and studied the components of each system carefully. As yet no correlations between these findings and those reported here have become obvious.

An important application of the separation of the resting and activity oxygen consumptions may be found in a consideration of the remarks of Burk (1937), *viz.* that in many cases the entire evidence for resynthesis of carbohydrate and the Meyerhof cycle disappears if the *total* oxygen consumption during recovery rather than the

excess above the original resting rate is used in calculation. However, if the resting respiration is a qualitatively different system, it becomes more justified to assume that only the extra oxygen is involved in recovery. Another comment would be that such a separation necessitates the view that stimulation of the muscle initiates a chain of new metabolic events rather than or in addition to "a raising of the barrier between metabolic and contractile mechanisms" (Hill, 1938). Among these new events would be a throwing into activity of the cytochrome-cytochrome oxidase system either directly or by providing it with substrate.

Also of interest in the light of such a qualitative separation of systems is the respiration of iodoacetate-poisoned muscle. Since the work of Lundsgaard (1930) it has been clear that the lactic acid production of muscle could be completely prevented by IAA without much effect on the resting respiration. The range of concentrations in which this is possible is small, but the essential observations have been repeated many times. Recently it has become clear (Shorr, Barker, and Malam, 1938; Stannard 1938b) that the respiratory quotient of the respiration remaining after IAA has poisoned out the glycolysis is essentially the same as that of untreated resting muscle. Recently Barker and Shorr (1939) have eliminated many alternatives and shown beyond a reasonable doubt that IAA tissues whose lactic acid production has been completely inhibited can still oxidize carbohydrate. It was mentioned above that the R.Q. of resting frog muscle treated with sufficient sodium azide to completely inhibit the activity respiration is approximately unity (see page 397), thus indicating that the resting system may still be utilizing carbohydrate. The point to be answered now is whether or not the respiration remaining in IAA muscle represents the system identified here with resting muscle. If so, then the persistence of oxidation of carbohydrate in the absence of lactate formation is not conclusive evidence that the Meyerhof cycle does not function in the system associated with activity. It is true that Wright (1932) showed that muscles stimulated electrically after treatment with sufficient bromacetate to prevent lactic acid formation exhibited an excess oxygen consumption after stimulation equal to approximately 70 per cent of that seen in normal muscle. Also Hegnauer (1931) found that extra tension and oxygen consumption occurred in chemical contractures in spite of IAA poisoning. Observations of this nature are often interpreted as indicative of the part played by oxidation or oxidative resysthesis of phosphocreatine, and the correlation in these and other cases is good. It might be mentioned, however, that Saslow (1937) was unable to obtain perman-

ent increases in the oxygen consumption of muscles treated with both IAA and caffeine, a situation where the demand for excess oxygen is continuous, and the increases observed were not greater than those computable from utilization of the resting lactate content. The utilization of preformed lactate cannot account for the results of Barker and Shorr (1939) or Shorr, Barker, and Malam (1938). But the possibility that the substrate in these cases is being oxidized by a fraction of respiration not involved in the Meyerhof cycle should be eliminated before these data are finally established as negations of the Meyerhof concept.

It seems clear from the present summary that a study of the oxygen transfer mechanisms in intact frog muscle points to the participation of components not suspected from experiments with isolated enzyme systems. On the other hand, in spite of superficial contradictions, the major factors expected on the basis of *in vitro* experiments check well in the more difficult and limited experiments with intact tissue.

I wish to express my thanks to Professor Wallace O. Fenn for his continued interest and stimulating advice throughout this work, and Mr. W. B. Latchford for technical assistance with some of the experiments presented.

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DISCUSSION

Dr. Gemmill: I would like to ask Stannard if he has tried azide poisoning on the increased oxygen consumption due to added lactate.

Dr. Stannard: That particular experiment has not been done as yet. There is every reason to expect that azide would inhibit this increment.*

* Experiments completed since this paper was given show that azide not only inhibits the increased oxygen consumption due to added lactate, but with approximately the same relation between the concentration of inhibitor and the effect as described for muscle stimulated in various ways.

Dr. Gemmill: It seems to me, from the results given, that the system involved was a lactate system.

Dr. Stannard: Yes; in every case, except that of *p*-phenylene diamine, the azide-sensitive increments in oxygen consumption occur under conditions when increased lactate formation would be expected. Also numerous controls in these experiments show that extra lactate was actually produced. I lean strongly to the theory that the cytochrome oxidase-cytochrome system in frog muscle is the oxygen-transfer system for oxidation of lactate. That does not mean, of course, that the immediate substrate for this system is lactate, or that this system transfers oxygen to no other substrate either directly or indirectly. We still lack conclusive evidence that the cytochrome system is brought into activity only when lactate is produced, but it appears that when lactate is produced the oxygen transfer is mediated by this system.

Dr. Gemmill: Have you tried stimulation at slow rates during which lactate is not formed? I wonder if you could get the separation under these conditions.

Dr. Stannard: That has not been tried. It would be a possible means of answering the problem raised by your earlier question.

Dr. G. T. Cori: Does azide affect the mechanical properties of the muscle?

Dr. Stannard: Within the time necessary to produce complete inhibition of any increments in the O_2 consumption there is no marked change in irritability or strength of contraction. After the contracture appears there is, of course, a marked change in these properties. Experiments to date with hydroxylamine indicate that this substance has practically no effect on irritability or the mechanics of the contraction while producing much the same effects on the oxygen consumption. The mechanical effects with azide must be either delayed or secondary, for they appear much later than the effects on metabolism. Both the metabolic and mechanical changes with azide are completely reversible if the contracture is not allowed to continue too long.

Dr. Korr: Working with some mammalian tissues, I have confirmed your observations with respect to differences between cyanide and azide, and I should like to ask whether, in view of the fact that cyanide seems more effective an inhibitor than azide, you would say that all those in the past who have worked with cyanide have been misled in believing that it is a specific inhibitor only of the Warburg-Keilin system, or that it took out something else also.

Dr. Stannard: I would not like to generalize too much, but it seems to me that one should be very cautious and gather evidence by several means before concluding that a given oxygen transfer system is or is not present. That is one reason I thought it was necessary to understand what carbon monoxide was doing in muscle, since its apparent effect there was not what would be expected on the basis of participation of the standard Warburg-Keilin system alone. In view of the many things cyanide can do *in vitro* besides inhibit the cytochrome oxidase it seems to me unjustified to assume that it can be termed a specific inhibitor for cytochrome oxidase *in vivo*. It must depend on the particular organism or system used. Incidentally, there are cases in plant cells and tissues where cyanide and azide seem to be completely interchangeable.

Dr. Shorr: I wonder if the data you have presented might not throw some light on the observation that the anaerobic glycolysis of resting muscle is far below that of contracting muscle under the same conditions. Glycolysis in muscle appears to proceed in two steps or rather at two rates, in contrast with other tissues, in which anaerobiosis *per se* releases the glycolytic mechanism maximally, provided the concentration of substrate utilized for the production of lactic acid is optimal. Your experiments with azide furnish strong evidence for the existence of two mechanisms controlling oxygen consumption, one dealing with resting, the other with contracting muscle. It is therefore conceivable that the glycolytic mechanism may also be a two-fold one, and different for the resting and stimulated muscle. It would be interesting to test this conjecture. One approach suggests itself, on the basis of a coupling of oxidation and lactic acid resynthesis. This would consist in ascertaining whether the resting metabolism which is unaffected by azide could bring about the resynthesis of lactic acid formed by previous anaerobic exposure under resting conditions; and whether lactic acid produced by azide-poisoned contracting muscle anaerobically would fail of reconversion.

Dr. Stannard: I think the point raised is an extremely interesting and important one. The low glycolytic rate of resting muscle as compared with contracting muscle has, to my knowledge, never been satisfactorily explained. In tracing the two pathways of oxygen consumption proposed here, further toward the substrate end of the chain, this problem would surely be encountered. It will be important to know whether the oxidative substrate utilized by resting muscle has preparatory steps in common with that oxidized during or after contracture. The method you suggest might be very useful in investigating this feature and be of importance in determining if the processes set off by stimulation arise *de novo* from the very first step or differ from the resting processes only at more distal points (including

the method of oxygen transfer). It seems to me that the difference in rates could conceivably rest on either basis, although a complete separation of both glycolytic and oxidative mechanisms would perhaps have the virtue of simplicity.

Dr. Stier: Your differences between azide and cyanide give me encouragement to go ahead with a project in which we hope to produce substrains of bakers' yeast by use of various specific "inhibitors". By treating a parent strain just once with potassium cyanide we were able to start a new substrain of yeast. This yeast consumes about 80-90 per cent less oxygen in the presence of dextrose than the parent strain. We have tested it for some of the enzymes which are missing, and find, using the usual cytochrome oxidase tests, that that system is absent. Spectroscopic evidence indicates that the cytochromes are present. I should think it would be important to produce an azide substrain. We might thus have an opportunity of determining what enzyme systems are eliminated when sodium azide is employed as a respiratory inhibitor.

Dr. Barker: I wonder if you would elaborate for us on what you think the basal metabolism of your muscle depends? Would you consider the oxygen as going through some other heavy metal catalyst besides iron?

Dr. Stannard: I have no evidence beyond that quoted in the paper. This is one of the first problems in the program for future work. The possibility that the resting respiration represents a copper-containing enzyme system can be checked fairly easily. We really need to find an *in vitro* system which will be cyanide-sensitive and azide-insensitive. Since hearing Cori's paper, I have entertained the idea that the substrate for the resting respiration was hexosemonophosphate. There is some evidence in work by the Cori group that hexosemonophosphate is oxidized aerobically when lactate production is prevented by iodoacetate (*cf.* Cori, Cori and Hegnauer, J. Biol. Chem. *120,* 193, 1937), but I have no experiments to show whether this is true in azide-treated muscle. Also, the resting R.Q. must be accounted for.

Mr. Abrams: In regard to azide sensitivity, is it really necessary to assume the existence of two different enzyme systems? It is not an unusual phenomenon, when one does not work with pure isolated systems, to find that under the proper conditions an inhibitor may actually accelerate a reaction. Is it not possible that this is just another, though slightly different, case of that same phenomenon, and may not have any relation whatever to two separate systems?

Dr. Stannard: If I understand the question, you refer to numerous cases in which an inhibitor, *e.g.* cyanide, stimulates cellular respiration at low concentrations while inhibiting at higher concen-

trations. It should be pointed out here that the "stimulation" by azide occurs only in resting muscle in the middle concentration ranges and has been adequately explained, I feel, by the presence of a slight contracture. In muscle whose oxygen consumption is above the basal level azide acts almost exclusively as an inhibitor until the basal respiration is again reached. There is no evidence of stimulation in these cases. Unless we wish to assume azide stimulates *de novo* some system whose rate of oxygen consumption equals precisely that of resting muscle, I prefer to interpret the observations on the basis of two systems. Also, the "stimulation" here would have to persist even in 0.1 molar azide, a very different case from those in which the stimulation appears at low concentrations only. It also seems difficult to explain the sharp discontinuity on this basis. Finally, hydroxylamine effects the separation without any consistent evidence of stimulation even of resting muscle.

Dr. Barker: In our iodoacetic acid work on mammalian muscle we have not tried any stimulation to see whether, as you suggested, the iodoacetate would inhibit the usual extra oxygen consumption due to activity. The reason we eliminated the Meyerhof cycle concept of carbohydrate oxidation was that we did not get lactate production anaerobically. It is an interesting point that lactate added to iodoacetic acid poisoned tissue is oxidized quite normally, for a time, at least, so that the point of attack of azide would be expected to be quite different.

Dr. Stannard: Yes; I had no intention of proposing that azide and iodoacetate attacked at the same point. Azide must be attacking at the oxidase end, but it seems possible that the separation of the resting and activity systems occurs anterior to the points of attack of both iodoacetic acid and azide. With no azide, of course, added lactate could be oxidized even in an iodoacetic acid tissue. But if the separation occurs high in the glycogenolytic system then the residual respiration of both iodoacetic acid and azide-treated tissues might be due to this oxidation and the Meyerhof cycle apply only to the activity system. That is merely a suggestion.

Dr. Barker: Have you ever tried nicotine? Nicotine will inhibit the oxidation of lactic acid in brain and yet not prevent the oxidation of carbohydrate.

Dr. Stannard: I hesitated to use nicotine because of the strong contracture it produces. However, it might be possible to use it in very low concentrations.

Dr. Barker: Is the nicotine contracture any worse than the azide?

Dr. Stannard: It is much worse at the high concentrations. I have not tested it at low concentrations.

SOME FUNCTIONAL CORRELATIVES OF CELLULAR METABOLISM

Herbert Shapiro

"Physiology as ordinarily understood is chiefly concerned in every case with the visible functioning of organs; biochemistry rather with the molecular events which are associated with these visible activities. I venture to think that productive thought in biochemistry in particular calls for the widest possible survey of life's manifestations. One of its ultimate tasks is to decide on what, from the chemical standpoint, is essential for these manifestations as distinct from what is secondary and specific. For any such decisions the necessary harvest of contributory facts must come from many diverse fields." F. G. Hopkins.

Introduction

Of the mechanisms by which oxygen is utilized by substrates in living cells, and the energy released, there have been numerous investigations. But now the question before us is, having thus set free energy, how is it applied to the service of those devices for which any particular cell is specialized, or to those general properties, such as selective permeability, which characterize all cells? It follows that there must be some sort of connection between these two general aspects of cellular function, for they are both lost when the cell dies; or, by inhibiting the energetic basis, certain of the other functions alter. In some few instances, as will appear in the sequel, some relations have begun to appear; in others, they are as yet merely parallelisms. Several facets of this problem will be taken up separately, viz., nerve asphyxiation, energy sources in muscular contraction, oxidations in relation to embryonic development, and luminescence and respiration within bacteria.

Through application of the concept of limiting reactions, Crozier and Hoagland and their associates have attempted to classify a wide diversity of biological phenomena, by means of temperature analysis. Throughout, this mode of attack has had the advantage of a theoretical rationale, and the results have been encouraging in view of the segregation of processes into certain discrete classes on this basis, and this has its analogy in the biochemistry of cells, in the relatively limited number of systems through which substrates are ordinarily oxidized.

Attempts to introduce order into a chaotic system by a simplified concept are sometimes vigorously opposed by those impressed with the general apparent disorder and complexity of the phenomena. Perhaps this is a salutary reaction. But the analytic method has always proved successful, especially so in a complex phenomenon where one may be fortunate enough in putting one's finger on some of the essential variables, and so disentangle them from other second and third order phenomena tending to complicate the picture.

On examining the so-called alpha frequency of human brain waves, with respect to temperature changes induced by diathermy treatment, Hoagland has adduced evidence for the metabolic basis of these electrical phenomena. Normal individuals and very early paretics have a μ value of 8,000, intermediate paretics 11,000, and advanced paretics 16,000 calories. In cases of advanced paresis, precipitation of iron has been observed histologically in the brain, and this would agree with the observation of a μ value of 16,000 calories for a system in which the iron-containing link is the slow one. What lends further attractiveness to this view is an *in vitro* analysis by Hadidian and Hoagland of the succino-dehydrogenase, cytochrome-cytochrome oxidase system, in which one or the other system can be selectively poisoned, and in consequence of thus being made the slow link, one or the other of two characteristic μ values may be shown to prevail. When succino-dehydrogenase is the slow link, 11,000 calories is the predominating value; when cytochrome-cytochrome oxidase is slow, 16,000 calories is the dominating value found.

Relation Between the Electrical Phenomena of Nerve and their Metabolic Bases

Relatively little work has been done on a correlation of the two, investigators ordinarily having confined themselves to one or the other. However, it is evident that the two are interrelated, and one is ultimately dependent upon the other for its existence. Thus we know that when a nerve dies, it no longer exhibits any of its electrical properties. This is rather a difficult and unexplored topic, and doubtless the answer to the general problem will resolve itself more clearly as the two components, mechanism of energy production, and mechanism of nerve conduction or muscle contraction, or cellular division, are in themselves more definitely elucidated.

One available approach is that through a study of the processes underlying the action current in nerve. Older studies (Gerard, 1927; Fenn, 1928) have indicated an increase in oxidative activity accompanying the passage of an impulse, though this was denied by Winterstein as characteristic of "natural impulses". The study of Gerard and Hartline, in which advantage was taken of light as an excitant of fibers originating in the eye, seems to have laid this ghost, though the improved microrespirometry of Schmitt has made a downward revision of the absolute values of in-

crease in uptake on conduction in frog nerve. When a nerve is placed in nitrogen or hydrogen it slowly fails or asphyxiates. An apparatus for the detailed study of this phenomenon was made by enclosing two pairs of electrodes in an all-glass chamber—platinum for stimulating, and calomel electrodes for recording. The entire apparatus could be immersed in a Dewar flask, and so kept at constant temperature for many hours. By stimulating the nerve repetitively with condenser charges and discharges through the nerve, and integrating the total action current by measuring the resultant ballistic throw of a sensitive galvanometer, an index of the total amount of electrical activity set up may be obtained. This measure does not distinguish between differential failure of the various fibres, or amplitude of the action current, or temporal spread of the wave. One general feature of removing free oxygen from a nerve in this way is that there is an ultimate

Fig. 2. Time for asphyxiation of nerve at various temperatures, in purified hydrogen (open circles) and nitrogen (closed circles).

decline in all of the electrical properties of nerve which can be measured, as indicated in Fig. 1. The first to disappear is the after positivity; following this, the action current. Both fall to zero. The relative change in injury potential on the other hand is small. The time taken to extinguish the action current is, like that of chemical processes in cells, an exponential function of the temperature (Fig. 2). No correction has been necessary for comparison of one nerve with another, as the nerves from different frogs were comparable, and gave asphyxiation times which fell along a smooth curve with respect to temperature. If a nerve be tetanized continuously during the asphyxiation process, the time for asphyxiation can be appreciably shortened. When oxygen is readmitted to the nerve after it has reached the point where it fails entirely to respond to stimulation, a recovery follows in the form of increasing action current as time goes on, in response to a standard stimulus. If the rate of this recovery process is taken at different temperatures, it again shows an exponential relationship. It is interesting to consider the results from the general view that the nerve, once deprived of oxygen, obtains energy for the setting up of impulses (in response to effective stimulation) from some kind of anaerobic decomposition; that the decomposition goes on spontaneously, and independently, regardless of whether stimulation is applied, but that the stimulation in itself removes a certain


Fig. 1. Differential sensitivity of action current, injury potential, and after positivity to oxygen lack. After positivity declined and disappeared most rapidly, action current followed, whereas injury potential was not extinguished during the experiment. All showed recovery in oxygen. Sciatic nerve of Hungarian bull frog, R. esculenta. Temp. 31° C.

amount of the available energy, and hence shortens the survival time. On readmission of oxygen, the train of recovery and synthetic reactions is again set up, and takes place on a grander scale than when the nerve is recovering from stimulation in air, where something closer to the nature of a steady state must prevail.

On plotting the results in the Arrhenius form (Fig. 3 and 4), a quite good fit is obtained, and the slope for the asphyxiation process differs from that for the recovery process, as is to be expected if the two events are different chemically. The critical thermal increment for the loss of conduction is 11,100 calories, that for recovery 28,000 calories. It does not seem unreasonable to suppose that we are here actually measuring the course of reactions which furnish energy—precisely how, is another and more involved problem —for the setting up of the electrical component of the nerve impulse.

Fig. 5. Arrhenius curve for absolute refractory period in nerve. Data taken from W. R. Amberson. $\mu = 18,400$.

Amberson (1930) determined the absolute refractory period in nerve at different temperatures, using a sensitive galvanometer. On replotting his results in the form of the Arrhenius equation (Fig. 5) a good fit is obtained, with a value of the critical thermal increment differing from those of the two processes studied above, viz. $\mu = 18,400$.

Gerard (1934) has set up a general scheme involving three different chemical reactions occurring during conduction, refractory period, and recovery, respectively.

A. During conduction, the breakdown of creatin phosphate yields decomposition products including an X substance which accelerates later reactions:

$$CrP \rightarrow Cr + P \ (+X)$$
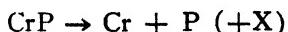
B. Resynthesis occurs during the refractory period with energy yielded by accessory reactions:

$$Cr + P + E \rightarrow CrP$$
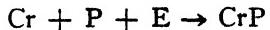
C. For recovery, certain oxidations occur, yielding energy and carbon dioxide:

$$F + O_2 \xrightarrow{\ (+X)\ } CO_2 + E$$
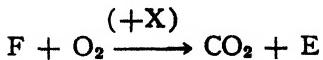
It is interesting to note from these other studies that temperature analysis yields results indicating three fundamentally different chemical reactions at the basis of these phenomena, as would be expected from the nature of the reactions postulated, although it does not give any specific information concerning the reactions involved.


Fig. 3. Arrhenius curve for extinction time of frog sciatic action current. $\mu = 11,100$.


Fig. 4. Arrhenius curve for rate of recovery of action current in oxygen in frog sciatic nerve, after asphyxiation. $\mu = 28,000$.

In further support of the chemical energy yielding notions adduced above from the results on nerve asphyxiation, may be presented certain of the analytical results of Gerard and Tupikova which are interesting parallels to the physiological results. When a nerve is deprived of oxygen, it loses phosphocreatine through decomposition. Tetanization during anaerobiosis accelerates the breakdown of phosphocreatine.

The Influence of Temperature upon the Dynamic Constants of Muscle

The relations between the mechanical and metabolic activities of muscles have been the subject of numerous investigations, many requiring delicate and laboriously contrived instruments for their elucidation. In a series of researches culminating in a monograph published recently by A. V. Hill, and which were to a great extent the fruit of much attention devoted to technical development, certain elementary facts have emerged, which throw light on the basis of the familiar experience that we can lift a light weight rapidly, and a heavy one only slowly. Fenn showed many years ago that lifting varying weights through a constant height results in a heat liberation which is in excess of the isometric heat at the same length, and more recently investigated the quantitative aspects of the force-velocity relations in amphibian and mammalian muscle, and found them to be susceptible of similar analytical expression. But now it is possible to combine the heat and mechanical measurements in one general expression with constants of known significance (Hill). Consider a muscle which is tetanized isometrically. During this isometric contraction, heat is given off by the muscle. If it is suddenly released, and thus allowed to shorten a given amount, it will give off heat in excess of that developed during isometric contraction, and this heat, the heat of shortening, is quantitatively related to the amount of shortening, as was earlier shown by Fenn. Now we may attach weights of various sizes to the muscle while it is shortening, and the muscle does proportionally more work in lifting a heavier load a given distance. However, the total heat of shortening is the same whether a light or a heavy load is raised. Hence the extra energy which the shortening muscle liberates may be classified as (a) the heat of shortening, which is proportional to the amount of shortening, and (b) the mechanical work done. In addition to these facts, it was also determined that the relation between the rate of excess energy liberation due to shortening, and the load lifted, was a linear one, *i.e.*, the rate at which excess heat was given off by the muscle due to its shortening was proportionally greater, the smaller

the load. These statements may be summed up by the equation

$$(P + a)(V + b) = (P_0 + a)b = \text{constant}$$

To show the derivation of this, let

$x =$ the distance, in centimeters, the muscle has shortened

$ax =$ the amount of extra heat liberated during shortening

$P_0 =$ the full isometric tension developed by the muscle

$v =$ the velocity of shortening, while raising a weight of P grams.

The energy liberated is then due to

ax, the heat of shortening

Px, the work done in lifting the weight P

and the total extra energy due to the shortening of the muscle is

$$Px + ax, \text{ or } (P + a)x.$$

If the velocity of shortening, V, is dx/dt, then the rate at which extra energy is liberated is

$$(P + a)dx/dt, \text{ or } (P + a)V$$

But since the rate of extra energy liberation is greater the smaller the load, and is zero at isometric length when $P = P_0$, it can be stated that

$$(P + a)V = b(P_0 - P)$$

b being a constant whose value is interpreted as giving an indication of the rate at which the extra energy is liberated. This equation is equivalent to the first one written above. P_0, a and b are called the dynamic constants of muscle since they characterize any type of muscle, in a quantitative manner, with respect to its metabolic and mechanical activity during contraction. Thus we can directly compare a turtle muscle, for example, with a frog muscle.

By setting the muscle in an appropriate mechanical device, all of this may be verified without making any heat measurements at all. Such an instrument was devised about ten years ago by Levin and Wyman. In it the muscle develops its full isometric tension (P_0), and then is allowed to shorten at a predetermined rate (V). The tension P it develops during shortening at this rate can be recorded and measured. Fig. 6 shows some curves describing the force-velocity relation, as obtained by this method (Shapiro, 1938), in their relation to temperature. The results are (1) that the equation is ap-
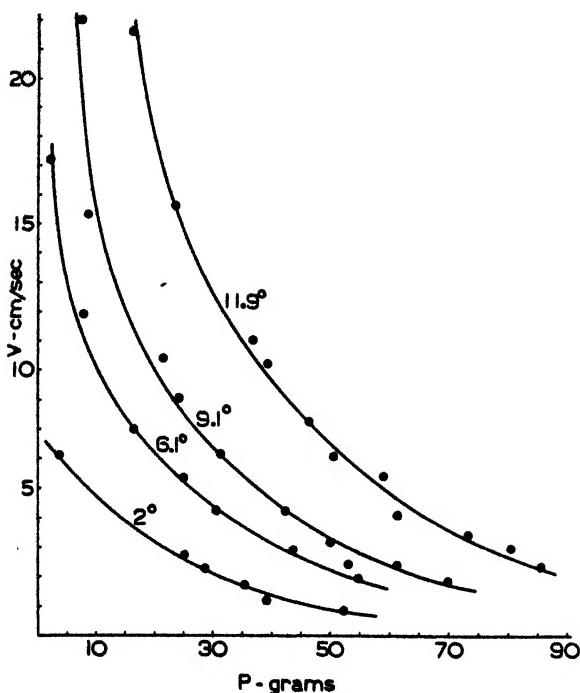
Fig. 6. Force-velocity curves of sartorius muscle of Hungarian bull frog, *R. esculenta*, determined with Levin-Wyman ergometer, at different temperatures. All data refer to the same pair of muscles.

plicable at all temperatures, (2) that the constant a/P_0 is relatively invariant with respect to temperature, (3) that the constant b has a high temperature coefficient. We saw above, in the derivation of the characteristic equation, that b was a constant of proportionality related to the rate at which energy was liberated. The interesting conclusion to be drawn from these experiments, after an examination of the exact mode in which b varies with temperature, is that the rate of energy liberation is controlled by temperature in a way that most chemical reactions are, *i.e.*, the higher the temperature, within limits, the more rapid the reaction. Hence at a higher temperature a muscle can give off energy more rapidly since the reaction is proceeding at a faster pace. It is to be presumed that at the basis of the contractile phenomenon there are two concatenated operating units, a chemical factory, which supplies energy, and a contracting mechanism. The way in which a muscle contracts is determined to a major degree by the rate at which chemical energy is fed from the chemical mechanism to the contractile. This hypothesis is substantiated, in particular, by the behaviour of these constants, to which a meaning may be attached. Other things being equal, a slowly contracting muscle should be one with a lower value of b.

On plotting values of b in the Arrhenius form, a good fit is obtained with a value for the critical thermal increment of 13,100 calories (Fig. 7). The constant b varies in muscles of different species, as might be expected; moreover, the seasonal condition of a frog may also thereby be reflected, although this has not been systematically investigated. Carbon dioxide, a common metabolic product, depresses the value of b, at all temperatures (Shapiro, 1939).

Keilin made the now well-known observation that spectroscopically all the cytochrome bands become visible during activity of the wing muscles of living insects. By means of a combined optical and oscillographic method, Urban and Peugnet have adduced evidence for the action of a yellow ferment and cytochrome-c during the contraction of a frog gastrocnemius in air. Millikan observed an appearance of reduced myoglobin bands during a tetanus in cat soleus muscle. None of these experiments, however, demonstrates conclusively the direct participation of these phenomena in the actual contraction process itself, involving the transfer of chemical energy to the contractile mechanism.

Thus far, the evidence for the direct dependence of two universal forms of specialized cellular activity, nerve conduction and muscular contraction, on a metabolic basis, appears fairly unequivocal. What is the situation in some of the other ubiquitous activities?

Metabolic Phenomena in Embryonic Development

In the amphibian embryo, the blocking out of the embryonic axis appears to be controlled in a region around the dorsal lip of the blastopore,
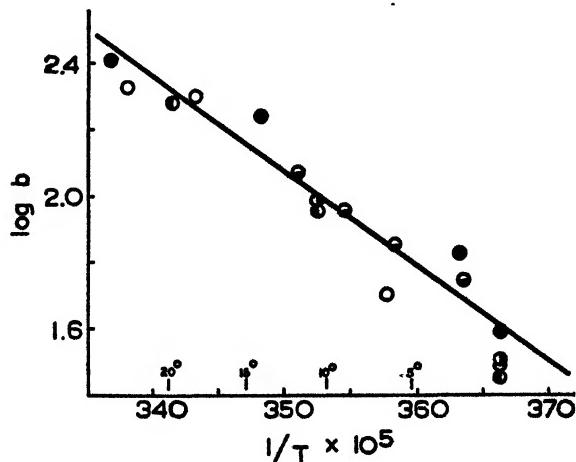

Fig. 7. Arrhenius curve for the values of the constant b (of A. V. Hill's characteristic equation) at different temperatures, as determined by mechanical (force-velocity) experiments. $\mu = 13,100$.

known as the organizer. The metabolism of this region was first studied by Brachet, who destroyed various regions of the embryo and observed a higher uptake when the dorsal lip region was left uninjured. In later experiments we were able to measure the uptake by that half of the embryo containing the dorsal as contrasted with the opposite side, in intact, unoperated embryos, by devising a suitable adaptation of a microrespirometer. In all cases, a higher uptake could be demonstrated for the organizer region than for the other half, and the average difference was 47 p.c. in its favor. Unfertilized eggs showed within experimental error a uniformity for both halves of the cell, this again for intact cells. Evidently it is this higher intrinsic metabolic activity which enables this region to control morphogenetic differentiation in adjacent areas. Whether this is a unique condition is problematical, and it may well be that it is only one aspect of a complex involving surface forces, metabolic products, and other factors as well.

To turn now to the metabolic events associated with the process of fertilization and the events leading to the first cleavage of the animal egg, we find that in sea urchins as a group there is generally a sharp increase in oxygen uptake on fertilization. Loeb stated that "one essential effect of the entrance of the spermatozoon into the egg of the sea urchin is the acceleration of processes of oxidation", and postulated a general accelerated metabolism on fertilization. Rogers and Cole observed also a sharp increase in heat production on fertilization. However, Loeb and Wasteneys were unable to demonstrate any change in respiratory activity on fertilization of the egg of the starfish. And as Rubenstein and Gerard discovered, the degree of acceleration of oxidations on fertilization is a function of the temperature, and at high temperatures, the effect may be much reduced. Whitaker also made the interesting observation that in the egg of the worm *Chaetopterus* there is a sharp drop in respiration upon fertilization. Hence we may observe in reply to the quasi-teleogical explanation that fertilization involves "an opening of doors", that in certain instances a few doors are also slammed shut!

In the sea urchin egg it appears quite clear from the alteration in values of critical thermal increment that the respiratory mechanism is changed, so that the Warburg-Keilin system becomes operative in the fertilized egg. Is it then the general rule that fertilization involves a sharp alteration in respiratiory mechanisms? In the *Chaetopterus* egg (Shapiro, unpublished data) the temperature coefficient of fertilized eggs is again different from that of unfertilized, though a chemical analysis of this remains to be undertaken. The starfish egg

stands out from the other echinoderm eggs in that there is no evidence from the respiration experiments for a shift in mechanisms on activation. The respiration on fertilization may show no change, or an increase varying up to twice the pre-fertilization rate (Shapiro, unpublished data), and over the temperature range 11° to 28° C., this response is invariant, indicating that in this particular type of cell, the process of development goes on without any profound alteration in respiratory mechanism—at least none that is detectable by temperature analysis, though it is by no means a necessary consequence that these shifts should be accompanied by considerably different critical thermal increments. The method of temperature analysis is a valuable and elegant one, but a good chemist considers it advisable to check his determinations by other methods. In a series of carefully controlled *in vitro* experiments, Sizer has shown the constancy of the value of the critical thermal increment, experiments which lend conviction to the reality of these constants; moreover, they demonstrate that in situations where the condition of the enzyme has been altered, this is reflected by a change in the value of the critical thermal increment.

Localization of Respiratory Activity within the Cell

The problem of the localization within the cell of the activity of respiratory enzymes is still almost completely open. Through an extended series of observations by Linderstrøm-Lang and Holter and their co-workers, we have a knowledge of the histological distribution of other enzymes, notably peptidase, which is more easily and precisely determined than other enzymes. It has been suspected of the respiratory enzymes that they are either adsorbed on or associated with discrete granules in the living cells. After triturating sea urchin eggs, and centrifuging them, Warburg found that the oxygen uptake resided largely with the granular portion. In those cases studied (marine eggs, amoeba) the proteolytic enzymes, however, were found to have a diffuse and uniform distribution. This was determined by Holter, who centrifuged the granules into one half of the cell, and then microdissected them away from the clear half.

It has long been a presumption of cytology that the nucleus was an active seat of oxidations within the cell, though the evidence for this derived, in the main, from rather dubious staining reactions. Other inferences (they were little more than that) were drawn from merotomy experiments on protozoa, in which the enuleated portion ultimately died whereas the nucleated part regenerated a complete organism, and carried on its life cycle.

Association of nucleus with cytoplasm does appear essential, however, for long-continued life of the cell.

By removing the rather large nuclei (germinal vesicles) found in amphibian eggs, Brachet could find no great difference between the nucleus and the cytoplasm with respect to oxygen uptake as measured with a microrespirometer. The gas exchange of the nucleus represented only about 1.5 p.c. of the observed uptake of whole intact cells. Warburg concluded that polyspermic eggs do not respire any more rapidly than monospermic cells.

By centrifuging in an isopycnotic medium, as Harvey and Harvey have done, a cell may be broken into two unequal parts, and these "halves" may in turn be fragmented into quarters. These fragments are not comparable to cellular or tissue "brei" in the ordinary sense. They are intact, living cells. In the egg of the sea urchin, *Arbacia punctulata*, the distribution of materials may be seen from the diagrams of Fig. 8. During centri-
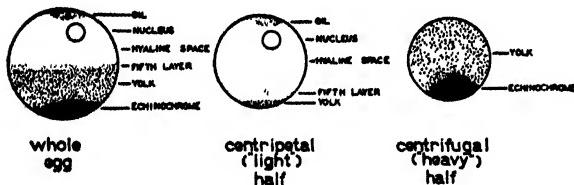

Fig. 8. Diagram to represent the composition and relative proportions of the whole egg of the sea urchin, *Arbacia punctulata*, and the "half" fragments obtained by centrifuging.

fuging the egg elongates into a dumbbell shape, and then breaks into two parts, a light half which rises toward the surface of the centrifuge tube, and a heavy half which is thrown to the bottom. The light half contains the nucleus, an oil cap, a layer of granules known as the fifth layer, some yolk, and occasionally a modicum of pigment which, if present, as a rule seems entrapped in the cortical region of the egg. Light fragments vary in the amount of retained red pigment, as observed macroscopically. Generally, the content was very low, so that a suspension of light halves would

appear quite colorless. The heavy half, devoid of a nucleus, contains most of the yolk and of the pigment (echinochrome) which is concentrated at the centrifugal end of the cell, and is not subsequently redistributed, but remains largely as a localized compact mass.

These two types of cell derived from a single cell are quite normal in all respects. They can be fertilized, or activated parthenogenetically, and will cleave and undergo a considerable amount of development, remain semi-permeable, and show a steady oxygen uptake over a period of hours. By repeated centrifugings, sufficient cells can be accumulated for measurement in the Warburg respirometer. The results are of some interest in connection with the problems mentioned above, and may be summarized in the following table.

Cell	Q_{O_2} (mm.^{3} O_2/10 mm.^{3} cells/hour)
Whole egg, unfertilized	1.49
Whole egg, fertilized	3.85
Light half, unfertilized	1.23
Light half, fertilized	3.49
Heavy half, unfertilized	2.81
Heavy half, fertilized	2.66

Thus the heavy half of the cell, containing most of the granules, and the pigment, but devoid of a nucleus, actually respires more intensely than the half containing the nucleus. These are the first observations of this nature where an attempt has been made in living cells to separate certain of the cellular components, and determine their effect on the net oxygen uptake. The whole egg, on fertilization, shows a several-fold increase in uptake. This has been known for many years, and appears to be characteristic of other sea urchins as well. The light half also shows a rise in metabolism of the same order, on fertilization, whereas the heavy half, when fertilized, remains practically unchanged.

What is the sum of the activities of the fragments in relation to the whole cell?

$$\frac{\text{Volume of heavy unfertilized half (84,700)}}{\text{Volume of the whole unfertilized cell (198,230)}} \times \text{ rate of heavy unfertilized half (2.81)} = 1.20$$

$$\frac{\text{Volume of light unfertilized half (116,750)}}{\text{Volume of whole unfertilized cell (198,230)}} \times \text{ rate of light unfertilized half (1.23)} = 0.73$$

Total respiration of light and heavy unfertilized halves, combined to the volume of the whole unfertilized egg — 1.93

Rate of whole unfertilized egg = 1.50

Difference between whole unfertilized and sum of unfertilized components $= -29$ p.c.

Similarly, for the fertilized cells:

Fertilized heavy halves $= 1.14$
Fertilized light halves $= 2.06$
_____

Total $= 3.20$
Rate of whole fertilized egg $= 3.85$

Difference between whole fertilized egg and sum of fertilized components $= +17$ p.c.

Now, in the unfertilized sea urchin egg, there is some evidence that the cytochrome system is largely inoperative, and is thrown into an active participation in respiratory events only after fertilization (Runnstrom). In a recent paper, Korr has concluded that "it is obvious from the data that a simple, non-ferrous carrier respiration, and respiration through the cytochrome indophenol oxidase system do not, *per se,* have different temperature coefficients or characteristics". On detailed reexamination and recalculation of Korr's data, it can be shown that the results for the sea urchin egg respiration not only obey the Arrhenius equation but these very good data yield strong evidence for the view of μ values as indicative of controlling enzyme systems. Crozier, in an unpublished analysis of Korr's data, has shown not only that the excess respiration due to fertilization obeys the Arrhenius equation with respect to temperature, and has a critical thermal increment of about 16,000 to 17,000 calories, a value known to be characteristic of iron-containing systems, but also the non-cyanide sensitive respiration obeys the equation yielding a μ value of 8,000 calories, while the effect of added pyocyanine also conforms beautifully to this type of temperature analysis. The respiration of the fertilized egg is cyanide sensitive.

Simultaneously proceeding processes are not to be expected to conform to the Arrhenius equation, but should yield curves concave upward (Crozier, 1924, 1939) when plotted. For example, by subtracting the corresponding points at various temperatures, for rates of oxygen uptake, using the published curve for fertilized eggs, one gets differences described by the Arrhenius equation yielding a μ of 17,500 calories, for that part of the respiration which is cyanide sensitive. A similar μ should be obtained by subtracting the data for unfertilized eggs from fertilized eggs. This value comes out 16,700 calories, a close enough agreement considering the nature of the calculations from enlargements of Korr's average curves through the data. The respiration of the non-cyanide sensitive system was likewise obtained by plotting the data for fertilized eggs in the presence of cyanide. This gives a μ of 7900 calories.

If the added pyocyanine effect is merely additive one should get a μ for this alone from Korr's data by plotting the differences between respiration of fertilized eggs + pyocyanine + KCN at two different concentrations of pyocyanine; and also the same result should follow for unfertilized eggs + pyocyanine unpoisoned by cyanide, at two different concentrations of pyocyanine. And in the same way the difference between fertilized eggs + pyocyanine, and fertilized eggs without pyocyanine, should yield essentially the same μ value. This turned out to be approximately 19,400 calories for all these calculations from the published figures. The value of 8,000 calories has frequently been found in association with respiration processes. A more common value is that of 16,000 to 17,000 calories which Hadidian and Hoagland have shown to be associated with an *in vitro* system of cytochrome-cytochrome oxidase. The value of 19,400 calories has occurred repeatedly with dehydrogenations (Gould and Sizer).

Since the light fragment behaves like the whole egg, in considerably increasing its respiration on being fertilized, it is to be presumed that it contains the Warburg-Keilin system, and that the heavy half is either devoid of it, or lacking one of its essential compounds. The heavy half contains the pigment and all the yolk, hence if part of the cytochrome system is adsorbed at the surface of granules, it might mean that there is a selective and quantitative redistribution of these granules so that only the enzyme-containing ones remain in the light half. But this does not appear likely. Of course, it might be held that in separating off, the heavy half became partially activated, but then an explanation would be required for the failure to show a more complete increase on fertilization, and for the complete lack of activating effect on the light halves; moreover, neither half cleaves or develops after centrifuging, unless fertilized or artificially activated. The detailed analyses of these cells are interesting problems which await further study with this material. As copper-protein systems may likewise be cyanide sensitive, the possible presence of such a system should also be checked.

In another cell, the optic nerve of the king-crab,

Limulus polyphemus, there appears to be homogeneity of structure along the length of the nerve, and yet a metabolic differentiation. This is a long uniform nerve running a distance of from 8 to 12 centimeters (depending upon the size of the adult) from the eye to the central ganglion, and giving off only a few very small branches. The non-nervous sheath enveloping the bundle of axons may be dissected away entirely, leaving the axons free. If the nerve is cut into five approximately equal portions, and each one is measured separately in the microrespirometer, it is found that oxygen uptake will continue at a constant rate over a period of hours (see Fig. 9), but that character-
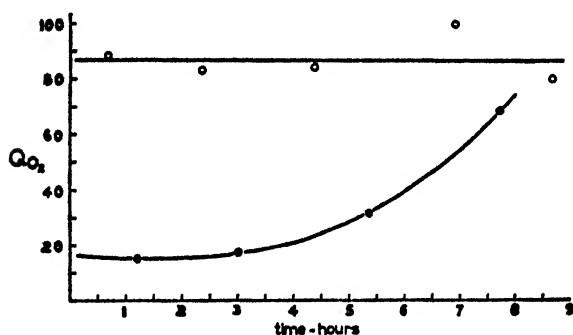

Fig. 9. Oxygen uptake by axons (open circles) of *Limulus* optic nerve, compared with that of nerve sheath. Oxygen uptake by axons proceeded at constant rate for many hours, whereas sheath respiration showed continuous rise after an initial uniform rate of uptake. Q_{O_2} = microliters of oxygen (corrected to 0° C.) consumed per gram of moist weight of tissue, per hour.

istic differences in rate of uptake are found, depending on the origin of the axon section. Those pieces cut from the middle region of the nerve show a higher rate of oxidation than the peripheral sections. This cannot be due to the contributions of the cut ends as the sections were made approximately equal. The localization of high respiration appears in both dark-adapted and light-adapted animals. A typical experimental result is given in the following table, A representing the section originally adjacent to the brain, and E that portion of the nerve closest to the eye.

Section	A	B	C	D	E
Q_{O_2}	111	140	119	106	73

Q_{O_2} = microliters of oxygen (corrected to 0° C.) consumed per gram of moist weight of tissue, per hour.

A similar result appeared in twenty-five other series of experiments under varying conditions, and over a temperature range of 18° to 28° C. It is intriguing to think that this physiological differentiation might play some part in the economy

of the passage of the nerve impulse, but there is no evidence for it. The results represent, however, a systematic exploration of the respiration of a single cell, the axon, along its length, with the unambiguous result stated above. Considered statistically, the number of permutations (5!) of the five regions is 120, *i.e.,* that is the possible number of ordered arrangements. The likelihood that any one of these ordered arrangements will appear fortuitously is only one in 120, and that it will appear so in successive experiments on different optic nerves, of corresponding low probability. This is brought into the discussion to consider the possibility that the situation may be entirely attributable to the chance occurrence of differences in activity of the portions of a nerve, so subdivided. If we allow the maximum to fall in either section B or C, then the number of permutations is smaller, *viz.,*

$$P = \frac{5!}{2!} = 60$$

So much for the differential localization within single cells.

Cell Permeability and Metabolism

Semi-permeability is one of the distinguishing characteristics of the living cell, and is lost upon death. Many of the theories of the structure of the cell surface are such as to presuppose the operation of purely physico-chemical and mechanical properties, without making provision for an energetic basis in the picture.

There are a number of interesting correlations between metabolism and permeability, illustrated most clearly in marine eggs. Lillie showed quantitatively in 1916 that the *Arbacia* egg increases its permeability to water by several fold, on fertilization, and this parallels the alteration in oxygen uptake in similar circumstances. The starfish egg was reported by Loeb and Wasteneys to be unaffected in its oxygen uptake as a consequence of fertilization, and Lillie could determine no appreciable change in water permeability on fertilization. These results in themselves might indicate that water permeability and cellular metabolism show a close parallelism, but in other forms which the author has investigated (unpublished results) this does not appear to be the case. There is likewise a parallelism between the calculated electrical capacity of the cell surface, and water permeability, again. The unfertilized *Arbacia* egg has a capacity of 0.86 $\mu f/cm.^2$, and on fertilization this rises to 3.3 $\mu f/cm.^2$ (Cole and Curtis). Other experiments may be cited which might at first sight indicate a complete independence of permeability and metabolic events. Hunter could

observe no change in the permeability of beef erythrocytes to lipoid insoluble non-electrolytes, ammonia, or acetic acid on altering the environment from an aerobic to an anaerobic one. However, one need not necessarily conclude that semipermeability is a property independent of oxidations. There may be reserves of different magnitudes and availabilities in the various types of cell, which contribute to the maintenance of cell structure, and which may be able to withstand anaerobiosis for some length of time. Removing oxygen from a nerve still leaves it with power to conduct, but this is eventually dissipated, and I have tried to show how it may be caused by the running down of some chemical reaction.

Now we come to the final aspect of the general subject.

Luminescence and Respiration in Bacteria

One of the more inscrutable phenomena of biology has been that of luminescence within living cells, its precise function being difficult to ascertain. It has been suspected, among other things, of being concerned with cellular respiration, though adequate proof of this is still lacking. The concentration of oxygen required for maintenance of full luminescence in bacteria is quite small, whereas the concentration necessary for a light intensity just perceptible to the completely dark-adapted eye is extremely low—so attenuated, indeed, that these organisms serve as useful indicators of the presence of oxygen in solution.

By determining the variation of light intensity with oxygen pressure, it may be shown (Shapiro, 1934) that the form of the function is such that it may be described by one of the simple adsorption formulations of Langmuir, viz.,

$$k_1 p (1 - \theta) = k_2 \theta$$

where p = gas pressure, θ = fraction of the surface covered by adsorbed molecules, $1 - \theta$ = fraction of the surface which is bare, and k_1 and k_2 are constants. The reactions occurring in the emission of light in a bioluminescent reaction in vitro involve the catalytic oxidation of luciferin by luciferase to oxyluciferin (Harvey, 1935):

luciferin (LH$_2$) + ½O$_2$ ⇌
oxyluciferin (L$_o$′) + H$_2$O
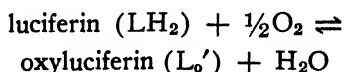
The luciferin functions as a receiver of the energy of oxidized luciferin, and thus has its energy level raised; this state is indicated by a prime (′). Light is emitted upon return to the normal state.

L$_o$′ + luciferase (A) ⇌ L$_o$ + A′
A′ ⇌ A + hν
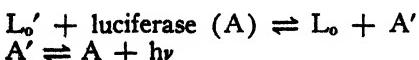
The adsorption of oxygen upon the enzyme luciferase is probably a fast reaction as are also the reactions involving a change of energy level. Here the velocity is dependent upon the luciferin and oxygen concentrations. The oxygen, present in the form of oxygen-enzyme complex, will be adsorbed to a degree depending upon the pressure and will vary as

$$\frac{k_1 p}{k_2 + k_1 p}$$

Thus the rate of the slow reaction is controlled by the fraction of the surface covered. The velocity of reaction ordinarily depends upon the fraction of the surface which is covered by adsorbed atoms or molecules. Enzymes are generally considered as colloids upon whose surfaces reactions take place. In his studies upon the kinetics in vitro of the bioluminescent reaction, Amberson made the fundamental assumption that the light intensity at any instant is a measure of the rate of the reaction, or more specifically of the rate of oxidation of luciferin. Hence we may write Rate = k intensity and from what has been stated above it may be shown that

$$\text{Rate} = \frac{100 \, p_{o_2}}{k + p_{o_2}}$$

which is equivalent to the adsorption isotherm. Thus the intensity of the luminescence in the living cell may be considered from this point of view as controlled by the fraction of the surface of the enzyme covered by oxygen, which in turn is a function of the partial pressure of the gas.

Having thus set up an intellectually and experimentally satisfying scheme of the luminescent reaction, we still have to face the more difficult question of its role in cellular energetics. Is it merely a permanent guest, or does it pay for its keep, in part? Using the fresh water luminous bacterium, Vibrio phosphorescens, Root studied both luminescence and respiration in the same cell as a function of temperature. The luminescence had a μ value of 20,000 above, and 27,000 below a break at 18°C. The oxygen consumption had a μ value of 15,000 above, and 22,000 below a break at 16.5° C. Thus from temperature analysis alone it might be inferred that the two processes occur at different foci in the cell. It is instructive to plot together (Fig. 10) the variation of respiration and luminescence in relation to oxygen pressure. The respiration curve represents the data on a marine form of luminous bacterium (Shoup), but this is very likely not too different from that of the fresh water form, whose luminescence/oxygen depend-
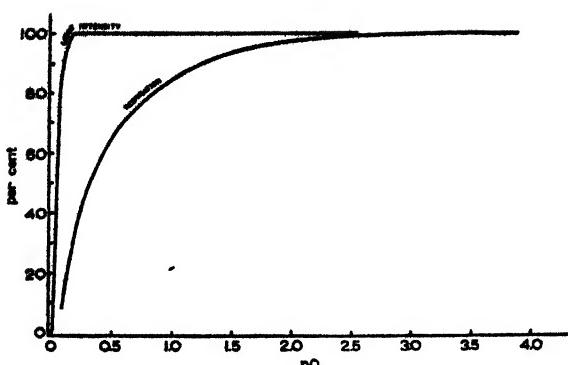
Fig. 10. Light intensity of fresh water luminous bacteria, and respiration by marine luminous bacteria, as determined by oxygen pressure.

ence is given in the other curve. The respiration begins to decline below 2.5 p.c. of an atmosphere of oxygen, whereas the light intensity is independent of oxygen pressure until it has fallen to a value of 0.14 p.c. (1.06 mm. Hg), where dimming begins.

If the two processes are links in a chain the different temperature functions would indicate that the enzymes concerned are distinct, and this is to be expected, too, on *a priori* grounds. I once asked a colleague to explain the relation of luminescence to respiration, and the immediate facetious reply was, "when things respire they burn, and from combustion you get light". This is a rather disappointing and unilluminating explanation; moreover, it can scarcely be said to be true, even as an analogy. In luminous bacteria a diversity of agents may affect the two processes in either parallel or opposite directions, which would appear to be evidence for the independence of the two processes.

At high oxygen pressures, there is again a marked differential response of the two functions. Shoup has demonstrated that in pure oxygen at atmospheric pressure the bacteria cease to take up oxygen, while their luminescence is scarcely affected. To affect the light as markedly one must go to much higher pressures. Application of 1500 pounds does not alter the luminescence immediately; on the contrary, it begins to dim very slowly indeed, and it would probably require hours to completely darken the culture. The fresh water and marine forms differ in their response to high oxygen pressure. The effect does not appear to be completely reversible (Shapiro, 1933).

Just as the enzyme concerned with light production appears to be inhibited at much higher oxygen pressures than that restricting the respiration (which, according to Shoup, is eliminated by placing the bacteria in an atmosphere of pure oxygen) so it may well be that there are other

enzymes in living cells which are saturated or inhibited at much lower oxygen pressures than atmospheric.

In the isolated non-circulated frog heart exposed to oxygen at 70 to 80 pounds gauge pressure a deleterious effect was observed (Bohr and Bean) in which the pace-setting mechanism was affected more rapidly than the contractile mechanism. This may involve a poisoning of the respiratory mechanisms, as Librecht and Massart observed a complete inhibition of fresh succino-dehydrogenase activity under high oxygen pressure.

Doubtless there is a pathway with its own detailed system by which oxyluciferin is again reduced, but operationally we take this to be a system acting in parallel with the respiratory mechanism.

There are certain properties of the cell, such as the low interfacial tension between oil and protoplasm, which appears to be a matter of the adsorption of protein at the oil-protoplasm interface, and a purely physical phenomenon not essentially affected by metabolic events. Thus, the interfacial tension between oil and protoplasm within the living mackerel egg (Harvey and Shapiro) is not significantly altered during development from the unfertilized egg to the 33 hour embryo.

Summary

The general purport of this discussion has been to introduce instances of a definite concatenation between metabolic processes in the cell and certain generally distributed cellular activities, and to emphasize that certain of these activities may be more profitably examined in the light of their metabolic basis. Experimental evidence has been adduced to show the nature of this relationship in nerve conduction, muscular contraction, and embryonic development.

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DISCUSSION

Dr. Bernheim: Will you describe the apparatus with which you measure the oxygen uptake of both halves of an intact cell?

Dr. Shapiro: It consists essentially of two capillaries in communication with a middle tube containing the frog egg or gastrula. An index drop is placed in each capillary and the rate of inward movement toward the egg or gastrula is a measure of the rate of oxygen utilization.

Dr. Pincus: How do you account for the difference between the unfertilized and fertilized frog eggs? No matter how you rotate the unfertilized egg, do you get no localized difference in oxygen uptake?

Dr. Shapiro: In the case of the unfertilized frog egg, the egg was so oriented that the vegetal half was on one side and the animal half on the other. In another series, the egg was symmetrically placed in the tube with equal quantities of animal and vegetal regions exposed to both capillaries. In neither case was any significant difference obtained. The purpose of these two series of experiments was to determine whether the yolk region was less active than the protoplasm-containing region with respect to oxygen uptake. The protoplasm (accepting the customary cytological interpretation of the word, which is used here for purposes of discussion and definition of the region referred to), however, constitutes a relatively small proportion of the animal half, hence small differences might be masked. Other orientations than those mentioned were not tried. In the case of the fertilized eggs, we were studying advanced stages, *viz*: gastrulae, and we interpreted the difference to be due to a higher oxidative rate of the dorsal lip region rather than to a larger absolute quantity of more highly respiring tissue. We are prepared to accept the other conclusion provided convincing evidence can be adduced. Some of the reasons leading to the view we took, and other relevant considerations are as follows.

1. The intact gastrula was used, whereas in all other experiments excised portions of the embryo were measured. In the latter a certain amount of development occurs during the experiment, and it is possible, though by no means a necessary consequence, that freedom of the regions from each other and their mutual effects of dominance or repression, which obtains in the intact embryo, may lead to more equal rates of cell division or other processes lying at the basis of requirements for cellular oxygen consumption.

2. The difference between the two hemispheres was found clearly in every experiment. The figures did not come from averaging experiments in which in some cases equal activity might be found in dorsal lip region and ventral ectoderm region, or even where measurements might indicate a reversal of activity, dorsal lip being lower than ventral ectoderm. When the experimental results show the variability of the type just mentioned, it is difficult to detect relatively small differences, and very large numbers of experiments must be done if a statistically significant difference is to be demonstrated.

3. There is the matter of units in which to express the observed O_2 uptake. Q_{O_2} has been generally expressed in terms of wet weight, or dry weight, or nitrogen content of the tissue. Other

variables may occur. Depending on how this is expressed, the values for Q_{O_2} may vary depending on relative content of moisture, nitrogen, etc.

4. Brachet's early experiments involved destroying various regions of the intact gastrula, and observing the relative decline in O_2 uptake. He found that destruction of the dorsal lip region caused a more marked reduction in observed oxygen uptake than destruction of other regions, indicating a higher intrinsic oxidative activity of the dorsal lip region. Also, Hartwig and Fisher (Biol. Zbl. *58*, 567, 1938) have observed a 20 p.c. greater oxygen uptake by dorsal lip as compared to ventral ectoderm.

5. Brachet and Shapiro observed a 47 p.c. greater O_2 uptake, on the average, for the dorsal hemispheres, than for the ventral one. This would presumably be a minimum value, owing to the possible tendency to equalization of the rates through contributions of the right and left sides to each other, by diffusion through the embryo. The actual oxygen uptake of yolk has not been determined separately, but it is not likely that it is inert metabolically, *i.e.* that its respiratory rate is very low. As a matter of fact, Brachet and Shapiro showed that in unfertilized frog eggs, which are largely yolk, values of Q_{O_2} were obtained which were of the same order of magnitude (somewhat less) as that for the ventral hemisphere of the developing embryo, on the basis of wet weight. This would agree with the notion that the actively dividing cell layers are bringing about the increased respiration. Histologically, the proportion of yolk cell layers is fairly large. Taking the oxygen uptake of the yolk as not negligible, and assuming a balancing out of the amount of blastocoel and gastrocoel cavities on both halves of the embryo as oriented in the respirometer, the observed uptake of the ventral ectoderm and dorsal lip tissues may in both cases be considerably diluted by the uptake of the yolk. Hence the real differences between respiratory activity of dorsal lip and ventral ectoderm hemispheres, which was observed as 47 p.c., may mean a still greater difference between dorsal lip and ventral ectoderm tissues (on the basis of moist weight). It is of interest in this connection that the embryos were placed in the respirometer at an early dorsal lip stage; hence the infolding was of only slight extent at the beginning of the experiment, and increased greatly during its progress (measurements of oxygen uptake were made over periods of seven to eight hours). If the excess respiration on one side were to be attributed to the simple increase of "actively respiring" layers it would be expected that the figures for Q_{O_2} should show a large rise on the dorsal lip hemisphere during the course of the experiment, when considerable infolding occurs. The data showed an average increase of

25 p.c. on the dorsal side and 18 p.c. on the ventral side over a six hour period.

6. An interesting aspect of this whole problem has to do with the theories of cellular growth and division and embryonic differentiation proposed by Rashevsky. Since the action of the organizer (and perhaps other substances on which it acts in turn) may be such as to bring about the proper balance of inwardly and outwardly diffusing substances, and surface relationships, as postulated by Rashevsky, it may well be that the embryonic form and movement is the net result of a dynamic balance of these several forces, and that absolute magnitudes are not the only determinants of these phenomena, but rather it may be also their functional interrelations.

Dr. Davenport: Did the enucleated part, which underwent division, as a whole increase in volume during development to the blastula stage?

Dr. Shapiro: The volume changes of half and whole eggs during embryogenesis have not been studied in detail, but their transformations are very much alike. The development of the fertilized halves parallels that of the whole egg, although the cleavage of the heavy half is retarded.

Dr. Davenport: Is there no evidence of any real growth of the mass, but merely a differentiation?

Dr. Shapiro: So far as I am aware, a quantitative study of the mass of the developing heavy half has not been made. However, the development of even the whole egg is difficult to carry beyond the pluteus stage, in the laboratory. The behavior of halves and whole eggs is quite similar in development, though the embryos from the half eggs are, of course, smaller. E. B. Harvey has made extensive studies of this and has numerous photographs illustrating these points. Cleavage of heavy halves may be abnormal.

Dr. Stannard: Have you been able to find, spectroscopically or otherwise, evidence of larger amounts of cytochrome or cytochrome oxidase in the light half? It would be a difficult, but interesting, problem.

Dr. Shapiro: I have not investigated the cytochrome or cytochrome oxidase content of the half eggs. It would be essential to demonstrate it for support of the notion that the increase in oxidative rate in sea urchin eggs on fertilization is due to the added activity of the Warburg-Keilin system.

Dr. Korr: In connection with that, I think it may be mentioned that numerous attempts have been made to observe cytochrome bands in the sea urchin egg, without success. One difficulty, of course, is the presence of the highly colored echinochrome. Two years ago J. K. W. Ferguson and I looked for the bands by reducing the echinochrome to the leuco form with sodium hydrosulfite. Although the spectroscope used was sufficiently sensitive to demonstrate reduced cyto-

chrome in many other cells, it could not detect it in the sea urchin egg even under these conditions.

In connection with the other question: an increase in respiration on fertilization in one egg-half but not in the other does not necessarily indicate distribution of cytochrome or even of cytochrome oxidase. It was my belief, when I wrote my paper on that subject, that, on fertilization of the sea urchin egg, cytochrome was activated or released from some complex or from some granules which perhaps broke down. More recent evidence from several sources indicates that the cytochrome system—that is, the whole Warburg-Keilin system—is brought in, not by an activation of cytochrome, but rather by a release of some substrate from its precursor which, in the presence of its dehydrogenase, reduces cytochrome, directly or indirectly, thereby "gearing" the Warburg-Keilin system with other respiratory systems.

Dr. Shapiro: The results on the relative oxidative activity of the light and heavy halves do not in themselves necessarily indicate an unequal distribution of cytochrome or of its oxidase. One or the other may be present only, or both may be present, but in an inactive state. Each of these possibilities should be tested. Is it possible that the yolk granules or reduced echinochrome interfered with the detection of the cytochrome bands? Have you attempted to observe them in the light halves, from which a large proportion of these cellular inclusions have been eliminated by being thrown into the centrifugal half?

Dr. Korr: No; this was done on whole eggs.

Dr. Graubard: I do not know about the cyto-

chrome, but the cytochrome oxidase is seldom found in eggs. For instance, in *Drosophila* eggs, which you can easily isolate, you do not find any cytochrome oxidase in the egg stage, but ten hours later you will find lots of it with the first appearance of the larva. As soon as the embryo can get out of the shell it has the oxidase, and lots of it, relatively speaking, but I do not know of any eggs that show it.

Dr. Shapiro: There are some interesting observations by Bodine and Boell on the developing grasshopper egg. They found that the major portion (80 p.c.) of the respiration of actively developing grasshopper embryos is cyanide and carbon monoxide sensitive, but the oxygen uptake of diapause embryos was unaffected by cyanide. The respiratory activity during diapause is reduced, it appears, through suppression of the cyanide sensitive respiratory mechanism.

Dr. Graubard: Is the cytochrome present in the egg? Has cytochrome ever been observed in eggs?

Dr. Korr: It is present in the sea urchin egg, fertilized or unfertilized, as indicated by the fact that p-phenylene diamine is oxidized in both cases, and at equal rates. I think this observation substantiates the belief that cytochrome is ready to act in the unfertilized egg, because, as Stotz, Sidwell and Hogness have shown, the oxidation of p-phenylene diamine requires both cytochrome (-b and -c) and cytochrome oxidase. So I certainly think they are both there, even though not functionally connected with the substrate-dehydrogenase systems.

A COLLOQUIAL CONSIDERATION OF THE PASTEUR AND NEO-PASTEUR EFFECTS

Dean Burk

Part A. The Crystallization of Contemporary Outlook

The Apprenticeship of a Pasteur Effect Expert. My first acquaintance with the Pasteur and Meyerhof series of investigations on metabolism was made some fifteen years ago while sitting on the lawns footing the Campanile at Berkeley, with copy in hand of Meyerhof's Chemical Dynamics of Life Phenomena. This fascinating little book, translated into beautifully simple and clear English, and a veritable New Testament of biochemistry, teemed, nevertheless, as does an Old Testament like Lewis and Randall's Thermodynamics, on every page and in every paragraph, with problems and questions decidedly beyond the grasp of an unhatched Ph. D. Nor was it of avail for me to move up to the higher climes of the Big C on the hills above, with the added inspiration of the scene before me—on my left Jack London's Oakland, where I was born; straight ahead in gorgeous sunset the Golden Gate which Sir Francis Drake had overlooked on a foggy morning; and to the right Mt. Tamalpais and Marin County, where I grew up, and constituting an earthly paradise of several hundred square miles of the most beautiful and varied scenery in the Universe.

At this time I could not see why Meyerhof should want to write an oxidation *quotient* rather than consider simply a numerator of decreased fermentation on the one hand and a denominator of respiration on the other; and so, quite overwhelmed by the subject, I wrote Professor Drummond in London, shortly before I was about to

hatch from the University of California, to ask if I might come to his laboratory on a National Research Council Fellowship to work, as suited him, either on photosynthesis (the subject of my Ph. D. thesis) or on biochemical nitrogen fixation (then, for me, a side-line). His warm welcome included a delicately worded suggestion that I avoid solar photosynthetic work in the city of London, and I can now testify that during the year 1927-1928 I saw no patch of perfectly clear blue sky appear over this very hub of the Universe between October 1 and April 1, and so I set to work on nitrogen fixation.

But at University College the presence of W. K. Slater, A. V. Hill, the Eggletons, and others stimulated anew my interest in glycolysis, and a work on the thermodynamics of glycogen-lactic acid breakdown was the result. Not until 1935, however, did I develop a concrete, analytical interest in the Pasteur phenomenon, when, at the International Physiological and Botanical Congresses in the U. S. S. R. and in Holland, I carried on many discussions with Tamiya (the first, in 1927, ever to see the Warburg *Atmungsferment*); Tamiya insisted that yeast did *not* synthesize carbohydrate from alcohol as Meyerhof had claimed. I vigorously asserted that surely Meyerhof would not make a mistake like that, though subsequently I was, of course, to change my mind (20). A few weeks later, in discussion with Quastel, Greville, and Knight at Middlesex Hospital in London, Quastel was quite insistent that the Lipmann experiments in explanation of the Pasteur effect had no real physiological basis. He said that there was not enough known, naturally occurring, oxidation-reduction catalyst present in real living systems. (How far we have come since 1935, with coenzymes now found of the order of tenths of a per cent.) Quastel cited the then recent work of Ashford and Dixon (2), in which mere addition of KCl was found to produce aerobic glycolysis in brain slices, and suggested that better clues as to the nature of the Pasteur effect might develop from following up this observation. Greville, evidently less emphatic and salty than Quastel, merely shook his head about the Lipman explanation, leaving to the imagination of his orectic hearers what thoughts and biochemistry were going on in his unsliced brain. Now, all this was very interesting because I was to drop in on Lipmann in Copenhagen in a few days, and I made up my mind to delve into both sides.

Lipmann, an F.R.S. of good sort (Früherer Rona Schuler), and like myself one of the Bloody Beggars Team working with Meyerhof in 1928,

met my train at midnight, and we talked until three in the morning about the Pasteur effect. Lipmann dismissed the "KCl"-Pasteur effect as probably irreversible, irrelevant damage to the delicate brain tissue. It was at this "bull-session" past midnight that I definitely dug my teeth into the problem of the Pasteur effect for good; I was now definitely enrolled as a graduate student in the subject. The next day we spent much time looking up some of the old Meyerhof data (*cf.* Table IV) and found some very healthy germs of suspicion regarding Meyerhofiana along lines laid down preliminarily by Kluyver in 1931 (71). Lipmann then sent me to Engelhardt in Moscow and finally to Kluyver in Delft, who put the finishing touches on my post-graduateship and awarded me the degree of P.E.D., upon which I felt qualified for public appearance on the question of the Pasteur effect, and here, after a few years of trying my spurs in the field, I am before you.

On Various Pasteur Effect Experts. I have commenced this talk with the foregoing personal narrative as to the sort and length of Pasteur effect apprenticeship I have served, in order to lay some background for the very broad outlook and position I am going to adopt in regard to the present status of the Pasteur effect and the Neo-Pasteur effects: a broad outlook which is very possibly not fated to meet immediately with that general acceptance that I am confident it ultimately will. For, as there are now a many-headed multitude of what may be called Neo-Pasteur effects, so there are many and various Pasteur effect experts. In the earlier sessions of this Symposium the whole room was full of them, particularly during the Kempner session. Parenthetically I wish to take occasion now to note the regrettable absence here at the moment of Michaelis, Ball, Barron, Shaffer, and King. Not all Pasteur effect experts are made, however, by serving such a long and somewhat hesitating apprenticeship as have I. Some are created *de novo,* from quite unsuspected clay; thus Ball did publicly but withal casually bring forth to us, on June 23rd at these sessions, what is probably one of the most concrete and explicit explanations of the, or a, Pasteur effect, or, at any rate, a statement as to its probable ultimate locus. Some experts are, indeed, self-appointed; others I have appointed (see below). By far the great majority, however, belong to a class which, vaguely aware of the Pasteur effect and problem, rather accidentally obtain some sort of Pasteur effect, often with some special organism and set of conditions, and announce boldly, not infrequently in *Nature* (or in the good old days, *Naturwissenschaften*), that here is *the* explanation of the Pasteur effect. It

is this human, indeed lovable, but mathematically-impossible-that-they-could-all-be-right class that we must be wary of. They are not to be regarded as like the Bander-log in Kipling's Jungle Book, who all hung together with, "We are the wisest in all the jungle. We all say so, therefore it must be true"; no, present-day Pasteur experts fall apart almost like the tigers in Little Black Sambo, each wishing with a single captured garment—experiment—to have the grandest explanation in all the jungle.

A Few Pasteur and Neo-Pasteur Effects and Explanations. It would take a seminar of many sessions to study through all of the various Pasteur effects—explanations, theories, views, definitions, reactions, mechanisms, schema—appearing in the literature, of which I have a collection of well over three score, most of them having come to light during the last three to five years. I have with considerable effort listed in Table I a baker's fifty of the classic effects or explanations [*cf.* (20)] along with a good many of the more common Neo-ones, and offer apology not so much for those either not mentioned or those in a sense overmentioned explicitly or implicitly, as for the brevity of the identifying catch phrases, which can scarcely in any instance be adequately descriptive. Some more Neo-effects could be derived from Table II on aerobic glycolysis occurrence, inhibition, and acceleration, but I believe Table I will suffice to give a very concrete impression of the expansive and expanding nature of our subject. Some of the Neo-effects could obviously be readily classified under more than one heading. In almost all, though not all, of the papers referred to in Table I some direct reference to the Pasteur effect was implicated by the respective authors.

One could discuss these Pasteuriana and many more at great length. If anyone should ever have occasion to refer to a Pasteur-Burk view I hope he will bear in mind to refer in one swoop to all of the (meritorious) ones above and many more that will by such occasion surely have been shown to exist, all comprehendable in terms of "O_2 inhibition of fermentative processes" (Table I, line 1). There is yet to be a Pasteur-Stern effect, and we are without fail reserving a place for that, or rather a Pasteur-Stern-Melnick "agent", when our colleagues from Yale isolate it, which I understand they are about to do with their modern Diogenes lamp in the form of a spectrophotometer. The agent is still such a rare bird, I also understand, that instead of throwing the customary salt on its tail they will use whiffs of carbon monoxide, in order the better to expose its gay chevrons to the beams of their searching lamp. It is to be hoped that in the excitement of their chase, the generalizing view of Barron, as broad

TABLE I.   A List of the Classic, and some of the Neo-, Pasteur Effects

Effect or explanation, etc.	Catchphrase
Classic (1860-1933)	
Pasteur (108) (Pasteur-Burk (135))	O₂ inhibition of fermentative processes.
Pasteur-Wortmann (147)	Partial Unitary, partial resynthesis, I/N ratio 1-0.
Pasteur-Pflüger-Pfeffer (108, 110, 111)	Intermediate product oxidized.
Pasteur-Meyerhof (94, 20)	Oxidative resynthesis cycle, intermediate disappearance *several fold greater than* oxidation.
Pasteur-Warburg (138, 139)	Reaction connecting fermentation and respiration (heavy metal catalysis).
Pasteur-Lipmann (82)	O/R inhibition of glycolytic ferment activity.
Pasteur-Kluyver (71)	Modern Unitary.
Pasteur-Blackman (13)	Partial resynthesis.
Neo- (1930-1939)	
Pasteur-Lipmann Variants	
Pasteur-Ball (7, 135)	Cozymase-pyruvate O/R inhibition.
Pasteur-Gemmill-Hellerman (59, 135)	Triosephosphate dehydrogenase inhibition (IAA, CuO, Hg compounds).
Pasteur-Rapkine-Trpinac (115, 116)	Triosephosphate dehydrogenase sulfhydryl inhibition.
Pasteur-Geiger (58)	Oxidized glutathione as Pasteur-Lipmann agent in brain.
Pasteur-Shaffer No. I (121)	Sulfanilamide O/R product.
Pasteur-Elliott-Baker (47)	Aerobic glycolysis acceleration by dyes of low O/R potential; inverse Pasteur-Lipmann.
Pasteur-Engelhardt-Shapott (49)	Aerobic glycolysis acceleration by dyes of low R/O potential; inverse Pasteur-Lipmann: hydrogen acceptor inhibition.
Pasteur-Michaelis-Runnström (99)	Thioglycollate muscle extract activation, Pasteur-Lipmann confirmatory?
Pasteur-Michaelis-Smythe (100, 101)	Hexosediphosphate suppression, yeast, anti-Pasteur-Lipmann, adumbrated Pasteur-Rapkine-Trpinac.
Pasteur-Quastel-Yates (114)	Non-oxidative, glycolytic ferment-inhibitor combination, anti-Pasteur-Lipmann.
Shunt-Loci	
Pasteur-Engelhardt-Barkash (50)	Hexosemonophosphate phosphorylation or oxidation.
Pasteur-Szent-Györgyi (134)	Triose reduction or oxidation.
Pasteur-Boyland-Boyland (14)	Triose shunt.
Pasteur-Turner (136)	Triose shunt.
Pasteur-Moruzzi-Moruzzi-Bartoli (103)	Triosephosphate competition.
Pasteur-Dickens-Simer (38)	Methylglyoxal shunt.
Pasteur-Shorr-Barker-Malam (9, 124)	Pre-acid intermediate shunt.
Pasteur-Potter-Elvehjem (112)	Pyruvate shunt.
Inhibitor-Accelerator	
Pasteur-Dickens (30, 32, 34)	Phenazine dyes.
Pasteur-Laser (78, 79)	Low pO₂, CO aerobic glycolysis stimulation.
Pasteur-Negelein (105)	Cyanide biological anaerobiosis.
Pasteur-Mendel-Strelitz (92)	Ferricyanide tumor specificity.
Pasteur-Chaix-Fromageot (21)	Sulfhydryl action, propionic acid bacteria.
Pasteur-Lennerstrand (76)	Aerobic removal of adenylic acid as PO₄ acceptor (decrease in AMP/ATP).
Pasteur-Krah-Eicholtz (73, 48)	Ferro- (not Cu-) catalytic agent.
Pasteur-Boysen-Jensen (16)	Respiration-fermentation separation, anti-Unitary.
Pasteur-Lundsgaard (88, 89, 90)	Respiration-fermentation separation, anti-Unitary.
Pasteur-Bumm-Appel-Fehrenbach (18)	Reduced glutathione.
Pasteur-Baker (4)	Anti-glutathione (Bumm-Appel-Fehrenbach) and anti-phenylhydrazine effects (Dickens).
Pasteur-Crabtree No. II (27)	X-radiation glycolysis inhibition at low temperature.

TABLE I.—Continued

Effect or explanation, etc.	Catchphrase
"Physico-chemical"	
Pasteur-Barron (10)	Integrated orientation of reactions.
Pasteur-Kluyver-Hoogerheide (72)	Cell O/R potential, surplus theory.
Pasteur-Shaffer No. II (135)	Enzyme polarization and separation from substrate.
Pasteur-Ashford-Dixon (2)	KCl inhibition.
Pasteur-Kempner (69, 68, 135)	Low oxygen pressures.
Pasteur-Belitzer (11)	Inverse pO_2 glucose effect.
Pasteur-Gaffron (135)	pO_2 on photosynthetic rate and induction.
Physiological	
Pasteur-Crabtree No. I (26)	Reversed Pasteur effect-glycolysis inhibition of respiration.
Pasteur-Dixon (41, 42, 43)	Carbohydrate utilization inhibition.
Pasteur-Dixon-Holmes (44)	Permeability.
Pasteur-Bumm-Appel (17)	Diversion into growth processes (low O_2 pressure).
Pasteur-Pett (109)	Cyanide yeast Pasteur effect abolition.
Pasteur-Davis (29)	Three type classes in bacteria, zero, negative, positive.
Pasteur-Stier-Stannard (132)	Ground, resting, yeast cells.
Pasteur-Waksman-Foster (137)	Lactic-alcoholic (double) fermentation.
Pasteur-Ludwig-Allison-Hoover-Minor (87)	O_2-sparing action depending on concentration of intermediate, inverse Pasteur-Dixon effect.
Syntheses	
Pasteur-Winzler-Baumberger (146)	Intracellular carbohydrate synthesis, yeast.
Pasteur-Clifton-Logan (23, 24)	Intracellular carbohydrate synthesis, *E. coli*.
Pasteur-Giesberger (61)	Intracellular carbohydrate synthesis, *Spirillum*.
Pasteur-Sprince-Stier (129, 131)	Higher carbohydrate synthesis inhibition, yeast.
Pasteur-Hoover-Allison (in press)	Intracellular synthesis Rhizobium.
Pasteur-Willstaetter-Rohdewald (144) ·	Higher carbohydrate synthesis.
Pasteur-Mirski-Wertheimer (102)	Higher carbohydrate synthesis.
Pasteur-Barker (8)	Glycogen synthesis, *Prototheca*.
Pasteur-Runnström-Sperber (117, 118, 119)	O/R-regulated storage mechanism.
Pasteur-Smythe (128)	Fat synthesis, yeast.
Pasteur-Euler-Hellström-Günther (52, 51)	Liver carbohydrate resynthesis, respiration-fermentation separation.
Pasteur-Bach-Holmes (3)	Liver carbohydrate synthesis, insulin.
Pasteur-Stöhr (133)	Liver glycogen synthesis.
Pasteur-Hegnauer-Fisher-Cori-Cori (65)	Muscle glycogen synthesis from hexosemonophosphate.
Pasteur-du Vigneaud-Irish (46, p. 365)	Acetyl derivative amino acid synthesis.

as any of those in Table I (though by no means as all-inclusive as the writer's), will not be lost sight of:—

"The link between fermentation and respiration may be produced at the different steps in the series of reactions of carbohydrate metabolism which in fermentation end either in lactic acid or alcohol. One of these steps may be the oxidation of hexose phosphate; another may be the oxidation of trioses through coupled oxidations with the reversible C₄ dicarboxylic acids (Szent-Györgyi) or through the reversible reactions in citric acid oxidation (Krebs and Johnson). The link may occur also after the formation of pyruvic acid...."

"It is at once apparent that the *orientation of reactions* will depend on a *variety of factors*. The number of oxidation-reduction systems making up the series will determine the degree of complexity of the Pasteur reaction; the orientation will be affected by the oxygen tension, temperature, concentration of electrolytes, and hormones. (10, p. 228-229).

Pasteur himself spoke in like vein, but even more generally, sixty-three years ago (108a, p. 276), in what better words?: "It must be borne in mind that the *equation of a fermentation varies* essentially with the conditions under which that fermentation is accomplished, and that a statement of this equation is a problem *no less complicated* than that of the nutrition of a living being". (Writer's italics in both citations).

It is indeed remarkable how much interest has been stimulated and is growing concerning the Pasteur reaction during the last few years. I do not altogether understand it myself. Following the earlier review of Lohmann (86) in 1933, which was largely steeped with the now very shaky Meyerhof cycle theory, there appeared in

1937 three simultaneous, comprehensive, and quite independent, but extremely similar reviews, in England, United States and Russia, by Dixon (41), myself (20), and Engelhardt. The latter, co-editor with A. N. Bach of the leading Soviet journal of biochemistry, *Biochimia*, replied in these words when I sent him a separate of my paper,

"You will understand how much interested I was in your discussion when I tell you that during these last years I have come to be closely occupied with the Pasteur effect, so much so that my report (in Russian) to the All-Union Physiological Congress last October (1937) had for its object actually the same question you considered in your essay—a critical review of the present state of the problem of interaction between respiration and anoxidative metabolism. One can but wonder how closely similar our argumentation, criticism and conclusions—yours and mine—were on nearly all the principal points of the question concerned. I consider this problem to be one of the most important and tempting in modern theoretical biochemistry."

Some Timely Comments on the Original Basis of the Pasteur Effect: Pasteur's Concept (Fermentation). Meyerhof had studied certain aspects of the Pasteur phenomenon considerably before Warburg, but to my knowledge never referred to a Pasteur reaction or effect before Warburg did thirteen years ago, and indeed, Meyerhof has seldom done so since. Warburg was really the first to speak explicitly of the phenomenon and to give a definition of some sort, referring to it, in fact, as a reaction, "the Pasteur reaction". Recognition of this is rather important in view of the various miscellaneous and conflicting definitions that we meet with in the literature, and in particular Dixon's (41), of which more anon. Warburg is almost certainly to be regarded as the first to use an explicit definition or connotation. He said in 1926 (139, p. 241), "Atmung und Gärung sind durch eine chemische Reaktion verbunden die ich Pasteursche Reaktion nenne". His Pasteur reaction is the connecting reaction whereby respiration inhibits fermentation. As will shortly become evident, if not already so, this definition of Warburg must, nevertheless, be regarded as decidedly less general than Pasteur's concept as a whole. For one thing Warburg spoke of *respiration* inhibiting fermentation; Pasteur made the more general statement that *oxygen gas* inhibits fermentation, without specifying whether as a result of respiration or in some other manner direct or indirect. Warburg undoubtedly had the best of intentions in trying to describe the experimental Pasteur effect of Pasteur. In the background sentence immediately prior to that just cited in German he said, "If he (Pasteur) placed cells, which fermented under anaerobic conditions, in oxygen, the respiration which now began caused either the diminu-

tion or the disappearance of the fermentation" (139a, p. 246). But Pasteur did not specifically commit himself to the effect of aerobic conditions being limited to the ensuing respiration, and a great deal of work during the past few years, commencing mainly with the experiments of Lipmann (82), has amply shown that aerobiosis can be regarded as often exerting its effect independently of respiration or of variations in respiration. In view of the inadequate and conflicting definitions obtaining at present, surely it is desirable to make a fresh start, and what better than to go back to Pasteur himself, with his book full of explicit and implicit concepts (108)? Let us so proceed henceforth, with unstinted and due attention to the work and statements of this great man who made so few errors of fact or conception and whose book has so perfectly withstood the test of time these sixty years.

With recognition of the existence of the great variety of mathematically-impossible-that-they-could-all-be-right Pasteuriana indicated in the first section of this paper, the concept of a single agent, theory, or mechanism covering all instances obviously becomes untenable. There are not merely one, three, or ten Pasteur mechanisms, theories, explanations, etc. In contrast to this, however, we may regard the single expression "the Pasteur *effect*" as a shorthand description of a class of *experimental* phenomena varying widely in detail, but which may all be considered with Pasteur to involve "inhibition of fermentative processes by oxygen gas" (*cf.* Table I, line 1). The adjective fermentative is rather purposely employed here in place of the noun-adjective fermentation, and is meant not merely in the more restricted modern sense but in the sense employed in Pasteur's time, to cover not only alcoholic, lactic, butyric and many similar specific chemical fermentations but also a wide variety of phenomena making up the whole of anaerobic life itself. Pasteur's view here ascribed to him is derivable from many possible citations, *e.g.* (108a),

"....fermentation is a very general phenomenon. It is life without air, or life without free oxygen, or, more generally still, it is the result of a chemical process accomplished on a fermentable substance, i.e., a substance capable of producing heat by its decomposition, in which process the entire heat used up is derived from a part of the heat that the fermentable substance sets free. The class of fermentations, properly so called, is, however, restricted by the small number of substances capable of decomposing with the production of heat and at the same time of serving for the nourishment of lower forms of life, when deprived of the presence and action of air."

"....(fermentation is) continuous life in cells already formed." (p. 274).

"....fermentation—a chemical action, connected with the vegetable life of the cells—takes place at the moment when cells, ceasing to have the power of freely consuming the materials of their nutrition by respiratory

processes continue to live by utilizing oxygenated matters which like sugars or such unstable substances, produce heat by their decomposition. The character of ferment thus presents itself to us, not as being peculiar to any particular being or to any particular organ, but as a general property of the living cell. This character is always ready to manifest itself, and in reality, does manifest itself, as soon as life ceases to perform its function under the influence of free oxygen, or without a quantity of that gas sufficient for all the acts of nutrition. Thus, we should see it appear and disappear concomitantly with that mode of life; ....to this we may attribute all possible degrees of activity in fermentation, as well as the existence of ferments of every variety of form and of very different species. It may readily be imagined that sugar may undergo decomposition in a quite different manner from that of which we have just spoken, that instead of alcohol, carbonic acid gas, glycerine, and similar substances, it may yield lactic, butyric, acetic and other acids.'' (p. 114).

''....life can continue, under certain conditions, away from contact with the oxygen of the air, and since the altered nutrition is accompanied by a phenomenon which is of great scientific as well as industrial importance, we may divide living beings into two classes, *aerobian*, that is those which cannot live without air, and *anaerobians*, which, strictly speaking, and for a time, can do without air; these latter would be the ferments, properly so called.'' (p. 116).

''Ferments constitute a class of beings possessing the faculty of living out of contact with free oxygen; .... fermentation is the result of living without air.'' (p. 279).

Of the far-reaching principles developed by Pasteur the only important one not to stand the test of time was his view that fermentation processes were necessarily cell-bound:

''The chemical phenomena of fermentation are related essentially to a vital activity, beginning and ending with the latter ....fermentation never occurs without simultaneous occurrence of organisation, development, and multiplication....'' (108a, p. 278).

With due consideration of any modification called for by the last three quotations I believe that the best single-phrased definition of the Pasteur effect, reconstructed in essence from Pasteur's own writings, is something like "inhibition by oxygen gas of (vital or vitally-derived) fermentative processes." This quite general statement, which contains no commitment as to particular chemical fermentations or *their manner of measurement*, cover, in one guise or another, I believe, essentially all of the situations cited in Table I, although here and there a further sharpening of subtleties might be obtained by more liberal use of "quasi-", "anti-", "inverse-", "reverse-" and other similar modifiers as needed. Observe, once and for all, that the Pasteur effect as here given and defined is a purely *experimental* entity. It either *occurs (happens, takes place)*, or it does not, quite independently of what anyone may choose to think as to how or why it does or does not; it involves no immediate ideas of mechanism,

theory, or explanation.[1] The great variety of the latter submit in generality and understanding to the single expression, (Pasteur) *effect*.

Of the Pasteur effect we might say, with Pasteur himself[2] concerning science, that it is "built up of successive solutions given to questions of *ever-increasing* subtlety, approaching nearer and nearer towards the very essence of phenomena." (108a, p. 327) (writer's italics). The Pasteur effect obviously represents a state of affairs, a principle, not a specific contribution to science like the isolation of an enzyme. It is not a limited problem for which one obtains a solution, final or complete. Like the second law of thermodynamics, one does not solve it. One observes its operation under a wide variety of circumstances. With Engelhardt, quoted earlier, we may regard it as a problem in theoretical biochemistry, a kind of philosophy in itself.

Now, the foregoing very broad, generalized view of the Pasteur effect that I believe desirable to adopt in 1939 as well as in 1876 will very likely not meet with immediate acceptance upon the parts of some who have been thinking mainly in terms of, for example, the Warburg reaction or the Meyerhof cycle, or of many of the more recent formulations. In line with my outlook, however, I would affirm with Pasteur, "To bring one's self

[1] Though clearly establishing the existence of some sort of a relation between the fermentation and respiration or aerobiosis Pasteur wisely gave little pursuit to the mechanism, the circumstances and biochemical knowledge of the times not being propitious for the necessary quantitative approach. It is still fair to say in these times that the problem of the cause or mechanism of the Pasteur effect has never been more open, and certainly different explanations and combinations of explanations hold in varying degrees and under varying circumstances. A certain statement of Pasteur's on cause should be noted, however. Just as Warburg has said of cancer (139a, p. x, June 1930), "Interference with the respiration in growing cells is, from the standpoint of the physiology of metabolism, the cause of tumors," so Pasteur said concerning fermentation (108a), "The actual cause of fermentation is ....the fact of life without air" (p. 327). ".... cells become fermentative when their vital action is protracted in the absence of air" (p. 274). Pasteur thus regarded respiration in air and fermentation in the absence of air as alternative modes of life, the latter setting in when the former was cut off by oxygen deficiency. Pasteur recognized that fermentation was a far less energetic process than respiration, on a basis of equal substrate consumption, and he considered, as we do now, that the anaerobic breakdown is, in general though not always, greater than the aerobic breakdown partly because of energy requirements. This is perhaps as near to an explanation (rather more teleological than chemical) of the Pasteur effect as he came.

[2] or with G. N. Lewis, "The theory that there is an ultimate truth, although very generally held by mankind, does not seem useful except in the sense of a horizon toward which we may proceed, rather than a point which may be reached." (The Anatomy of Science, Yale Univ. Press, 1926).

to believe in a truth that has just dawned upon one is the first step towards progress;" though I do not readily follow him in the remainder of his statement "to persuade others is the second. There is a third step towards progress, less useful perhaps, but highly gratifying nevertheless, which is, to convince one's opponents." (108a, p. 316). In these two last matters, I must freely confess, I am none too like Pasteur in having a penchant for pedagogical projects of this sort; and, before attempting the third step at least, I should in any event assuredly await with unquixotic circumspection, the appearance of opponents, if any. After all, Table I is surely no imaginary windmill, and doughty warrior would he be who could look at Table I and see not the many but only two or three Pasteuriana. Almost certainly will some of the earlier-referred-to mathematically-impossible-that-they-could-all-be-right experts find it hard to give up newer individual fancies, not to mention the classical, but not perfectly general conceptions (Meyerhof cycle, Warburg reaction and the like), but time and the onrushing surf of Neo-Pasteuriana here exposed will, I have no doubt, overwhelm them like so many Canutes. Finally, I should say that some critics of my earlier review (20), including two colleagues (Hellerman and Gemmill) whose comments I could scarcely afford to overlook, complained that in that long and reasonably complete work I talked all about and around the Pasteur effect, and especially what other people thought about it, but I did not say definitely what *I* thought it was. I shall not now disappoint them again. Table I is my answer in general, and in particular: "O₂ INHIBITION OF FERMENTATIVE PROCESSES". To this might be added an interesting, seldom appreciated, (frequent but not invariable) sequel or corollary, to be brought out in the next section, "O₂ stimulation of anabolic syntheses (assimilation or growth)".

Pasteur's O₂ Stimulation Concept (Intracellular Synthesis). Pasteur was more constantly aware than many of us nowadays in recognizing that to maintain the well-being of a facultative anaerobe occasional exposure to aerobic conditions was highly beneficial or necessary, for either developmental, maintenance or recovery processes. (How many times do we all forget, in comparing the anaerobic and aerobic behavior of an organism, that anaerobiosis is often after a longer or shorter period harmful, irreversibly or reversibly, and that what occurs under anaerobic conditions may be largely eliminated aerobically by a series of quite indirect effects upon the organism as a whole: Pasteur-Barronism, but on a very broad scale). Pasteur said (108a),

".....yeast formed under these conditions (of perfect aeration) and subsequently brought in the presence of sugar *out of the influence of air* would decompose more *in a given time* than in any other of its states. The reason is that yeast which has been formed in contact with air, having the maximum of free oxygen that it can assimilate, is fresher and possessed of greater vital activity than that which has been formed without air, or with an insufficiency of air." (p. 254).

".....to multiply in a fermentable medium, quite out of contact with oxygen, the cells of yeast must be extremely young, full of life and health, and still under the influence of the vital activity which they owe to the free oxygen which has served to form them." (p. 246).

At first glance there might appear to be a touch of anti-Pasteur effect here, with prior exposure to oxygen being beneficial for fermentation, except that in this case the anaerobic fermentation might well be benefitted even more than the aerobic. This set of quotations further brings out, however, how broad a view Pasteur took as to the nature of the effect of oxygen, as well as of the nature of fermentation itself, as indicated in the first set of Pasteur quotations given in the previous section.

Quite apart from the beneficial influence of *temporary* exposure to O₂ on the general vitality of facultative anerobes and the rates of their fermentation processes, Pasteur made the important observation that *in general continued exposure to O₂ increased the amount (rate) and efficiency of the cell synthetic processes of assimilation and growth.* He said (108a),

"Free oxygen imparts to yeast an increased vital activity, inasmuch as under this condition it approaches the state in which it can carry on its vital processes after the manner of an ordinary fungus, the mode of life, that is, in which the ratio between the weight of sugar decomposed and the weight of the new cells produced will be the same as holds generally among organisms which are not ferments." (p. 253).

"In all beings that do not ferment the weight of nutritive matter assimilated corresponds with the weight of food used up, any difference that may exist being comparatively small." (p. 238).

"If we supply yeast with a sufficient quantity of free oxygen for the necessities of life, nutrition, and respiratory combustion, it ceases to be a ferment, that is, the ratio between the weight of the plant developed and that of the sugar decomposed is similar in amount to that in the case of fungi (3 to 4). On the other hand, if we deprive the yeast of air entirely it will multiply just as if air were present, although with less activity, and under these circumstances its fermentative character will be most marked; under these circumstances, moreover, we shall find the greatest disproportion, all other conditions being the same, between the weight of yeast formed and the weight of sugar decomposed." (p. 259).

"The alcoholic ferments can cause decomposition to an extent which, though variable, yet, as estimated by the weight of product formed, is out of all proportion to the weight of their own substance." (108a, p. 238).

Note, moreover, that Pasteur, who was in general much more concerned with ferment power than with ferment activity, was perfectly well

aware of the difference between rates and final amounts (or efficiencies) of fermentation and growth as a qualitative conception (even if he did not make so many quantitative measurements of rates compared to ferment powers); he was also well aware of the role played here by different degrees of aeration. Thus,

"....the power of a ferment is independent of the time during which it performs its function.... The power of a ferment is determined by the relation of the weight of sugar decomposed to the weight of yeast produced." (108a, p. 252).
"The fermentative power of yeast—which power must not be confounded with the fermentative activity or the intensity of decomposition in a given time—varies considerably between two limits, fixed by the greatest and least possible access to free oxygen which the plant has in the process of nutrition." (p. 259).
"....if free oxygen occurs in varying quantities, the ferment-power of yeast may pass through all the degrees comprehended between the two extreme limits of which we have just spoken (adequate aeration and complete anaerobiosis)." (p. 259).

Under anaerobic conditions both the rate and amount of sugar decomposing to cleavage product would be greater, considerably greater, than the sugar disappearing into cell material; thus, a fifteen per cent sugar solution might be practically completely fermented anaerobically to alcohol and carbon dioxide, with only a small fraction at most being converted to cell material. But under aerobic conditions essentially the reverse might obtain, practically all the sugar being converted to cell substance or (by oxidation) water and carbon dioxide, and none to cleavage product (alcohol); this, note, so far as *amount* of sugar decomposed. The *rate* of total sugar decomposition aerobically (in anabolism and oxidation) might be greater or less than anaerobically (in fermentation) depending upon conditions and the organism concerned; very generally considered, with yeast the anaerobic sugar disappearance would be faster than the aerobic, with common fungi the converse, and the same comparison could be drawn between unstimulated and stimulated muscle. It is a very common fallacy that Pasteur believed that oxygen decreased sugar consumption in general, whereas his view on this matter referred only to the sugar which went to form measureable cleavage product, and not necessarily to the sugar consumed in respiration or anabolism, a point to which we shall return with some vigor in the next section, in connection with the Dixon definition.

Pasteur generally found respiration several fold greater than aerobic anabolism, except mainly in the case of fungi, so that although he almost invariably observed increased amounts of cell anabolic synthesis (growth or assimilation) under aerobic compared to otherwise identical anaerobic conditions, he seldom obtained cases of aerobic anabolism greater than anaerobic fermentation

(even though aerobic anabolism *plus* oxidation might be), except again mainly in the case of some fungi. Nevertheless, in recent years, especially, there have been unearthed many cases where cell assimilation or synthesis in a variety of organisms can amount to a large fraction of the sugar consumed. Many of the "Syntheses" listed in Table I are good instances, especially among the first nine, and references (62), (85), (86) and (145) may also be cited. A good proportion of the cases now known concern the so-called resting cells, which, following the prominent work of Clifton and co-workers, have been shown not only to catabolize (as originally thought) but also to anabolize (assimilate) extensively. In some cases the aerobic assimilation is indeed actually greater than the concomitant oxygen consumption, reckoned, as it should be, in terms of sugar substrate consumed (*cf.* especially *Prototheca* (8) and yeast (146)). A recent paper as yet in press (S. R. Hoover and F. E. Allison: The Growth Metabolism of Rhizobium, with Evidence on the Interrelations between Respiration and Synthesis) is probably the most striking instance. Under aerobic conditions the organism involved, the root nodule bacterium *Rhizobium meliloti*, undergoes a growth reaction whereby about one fourth of the sugar consumed is converted to CO_2 and H_2O and the other three-quarters is converted into bacterial cell material, and no measurable extracellular organic products are formed, except possibly some gum under special conditions. The ratio of synthesis/oxidation of about 4 is superficially suggestive of Meyerhof oxidation quotients of the same value, but refers of course to sugar and not to cleavage product metabolism; in the youngest cultures the ratio may attain a value of 10. This paper, together with another recent one by the same authors (Trans. 3rd Com. Inter. Soc. Soil Sci., *A, 32* (1939)), represents one of the most thorough studies of resting organisms and cell synthesis extant, and clarifies many previously obscure issues involved in these phenomena.

In summary of this section, it may be said, among other things, that work since Pasteur, and more especially work in very recent years, has amply confirmed Pasteur in his view that cell synthesis and assimilation is almost invariably greater aerobically than anaerobically in the case of facultative anaerobes. The work of Sprince and Stier (129, 131) with yeast is one particular exception, but, as will be indicated later, not an important one in view of the constrained conditions employed.

Part B. The Contemporary Experimental Basis of the Pasteur Effect

On Criteria of Fermentation. The Pasteur effect of Pasteur, however experimental, was essentially

a qualitative concept, and was seldom put by him to more than semi-quantitative expression at best. Pasteur left largely to the future a detailed study of those quantitative "questions of ever-increasing subtlety" that involved, for example, variable oxygen pressure, rates as compared with amounts of fermentation, and in particular the exact method of definition and measurement of fermentation, in terms of initial substrate consumed, or of fermentation product formed (thus, in alcoholic fermentation, sugar consumed or alcohol formed, respectively). The last named subtlety is especially important at the moment in view of the point recently raised by Dixon, in his (I affirm) otherwise superb review, that the Pasteur effect defined in terms of O_2 suppression of substrate destruction renders superfluous and redundant definition in terms of O_2 suppression of fermentation product formed. He said, "we define the Pasteur effect as the action of oxygen in diminishing carbohydrate destruction and in suppressing or decreasing the accumulation of the products of anaerobic metabolism." (41).

Now measurements of the Pasteur effect during the past two decades have been carried out preponderantly in terms of cleavage product formation, whereas Dixon could count the experimental works based on substrate destruction on the fingers of one hand. Why, then, should the biochemical world at this date go over to his particular definition based on the use of substrate destruction as a more deciding, final and inclusive criterion than fermentation product accumulation?

quantitatively to far more than compensate for any sugar consumption decrease in fermentation proper.* This aerobic anabolic intracellular synthesis from *added* substrate such as extracellular carbohydrate could thus, when great enough, markedly falsify the quantitative measurement of fermentation proper made on a basis of added carbohydrate disappearing, instead of observed cleavage product formed. The experimental answers obtained by the two criteria would be different, the former being incorrect. For, as already indicated in the previous section, whereas Pasteur expected decreasd cleavage product formation in fermentation in air, he further expected, if anything at all, *increased* cell synthesis at the expense of added substrate (*viz.*, carbohydrate). This aerobic increase he did not consider to be fermentation proper, but to represent the aerobic and not the fermentative mode of life. Carbohydrate destruction here would be no true experimental basis for measuring the Pasteur effect, since there would be an aerobic increase (not a decrease as the Dixon definition would be looking for), and on this basis a Pasteur effect would be missed; this would not be true, however, on the basis of cleavage product measurements.

Second, considering cases not involving this superimposed intracellular synthesis, but only cases of fermentation proper, it is also possible for the carbohydrate consumption to increase aerobically while the cleavage products decrease, under certain conditions where the Meyerhof oxidation quotient,

$$\frac{\text{anaerobic cleavage product} - \text{aerobic cleavage product}}{\text{equivalent oxygen consumption}} = \frac{Q^{N_2}_{CO_2} - Q^{O_2}_{CO_2}}{Q_{O_2}/3} \tag{1}$$

Admittedly, mere weight of precedent alone, or even ease of measurement as is likewise involved here, should not pass on this matter. The really serious difficulty with the Dixon definition is that, despite explicit statement to the contrary (41, p. 432, last two sentences), it does not give a proper impression or experimental measure of those cases, now rapidly multiplying in number, wherein in oxygen sugar consumption increases while at the same time the cleavage products decrease or disappear. This situation, it may be noted, can come about in at least two quite different ways as foreshadowed in the previous section.

First, superimposed upon any fermentation proper which may be occurring aerobically (with the usual aerobic decrease in cleavage product accumulation) the organism concerned may, even quite incidentally' in relation to the fermentation, be utilizing sugar in the medium to build up various cell materials, possibly in a manner

is less than unity, as can commonly be the case in many stable animal tissues (*e.g.* liver, kidney, muscle fascia, thyroid, *cf*. Table VII and plant tissues. Whenever respiration is greater than enough to oxidize all cleavage products (Q_{O_2} greater than three times $Q^{N_2}_{CO_2}$), the consumption of added substrate can obviously increase in air with, nevertheless, cleavage products decreasing or disappearing ($Q^{O_2}_{CO_2}$ less than $Q^{N_2}_{CO_2}$).[8] Here

* *cf*. last section, and references 3, 8, 12, 23, 24, 51, 52, 61, 62, 65, 85, 86, 117, 118, 119, 127, 128, 129, 131, 133, 145, 146.

[8] It is very interesting to note that Ludwig, Allison, Hoover and Minor (87) have obtained excellent examples of this in alcoholic fermentation by plant roots and legume nodules and roots, and have shown further that whereas under *all* conditions studied the alcohol production decreased in the presence of air or oxygen, the aerobic carbohydrate consumption either decreased at times or increased at others depending upon the concomitant concentration of alcohol resulting either from prior accumulation or actual addition as substrate for oxidation (which oxidation these materials readily carry

it is the large oxygen consumption, instead of the cell synthesis as in the first case, which results in the *increased* carbohydrate consumption in air, and thus a missing of the occurrence of the Pasteur effect of Pasteur and Burk by the Dixon outlook.

The somewhat ambiguous and forced and at times experimentally incorrect treatment of certain (purely experimental, theory-free) Pasteur effects by Pasteur-Dixon definition, in the two foregoing cases where a Pasteur effect obviously obtains in the sense of aerobic cleavage product decrease as implicated by Pasteur and the great bulk of workers since, leads Dixon, when he is concerned with Meyerhof oxidation quotients of the order of unity or less,[4] to a number of fundamentally incorrect statements and implications throughout his (I repeat my affirmation) otherwise very superb, comprehensive, scholarly, valuable, constructive, timely (and within itself, self-consistent) review. In my opinion, the view of Dixon and undoubtedly of others who will have been impressed by the quantitative applications, as well as the experiment-provoking aspects of his definition, that the Pasteur effect does not occur with Meyerhof oxidation quotients less than unity, is (I am duty-bound to insist) fallacious, and if permitted to get loose and gain acceptance as a perfectly adequate fundamental conception would be de-

[4] It is not uninteresting in this connection that Sprince and Stier (129, 131) accept the Pasteur-Dixon definition but amplify it to mean ".... reduces both carbohydrate destruction and *carbohydrate synthesis*, suppresses etc. ...." They are among the few, if any other, workers to have obtained a *decrease* in aerobic intracellular carbohydrate synthesis (from glucose substrate): yeast was used and in this instance was very highly washed, hence probably deficient in riboflavin and soluble enzymes generally, and the results obtained represent merely the special and artificial case of the resting state with extreme depletion additionally imposed. As we have seen, Pasteur in general expected much greater cell synthesis under aerobic conditions.

out); at a given concentration or greater, alcohol was preferentially utilized in oxidation, and total carbohydrate consumption was *less* in air than anaerobically; below this concentration it was greater aerobically. Personally, on the basis of the invariable aerobic cleavage product decrease, I am satisfied to consider this a case of Pasteur's Pasteur effect operating throughout: the true fermentation measurable cleavage product accumulated always decreased aerobically even though the sugar consumption at times increased or at times decreased as a function of competitive preferential oxidation of cleavage product. The Pasteur effect here is unquestionably best measured, if one is looking for a measure, in terms of the observed cleavage product disappearance rather than in terms of the observed total substrate disappearance, which gives in this case two different answers, not uniformly one. In considering the Ludwig-Allison-Hoover-Minor data as well as in the discussion of this and the previous section, it is desirable to bear in mind that glucose can ordinarily be a substrate for fermentation, oxidation, and assimilation, whereas a cleavage product such as alcohol or lactic acid can usually be a substrate for oxidation only, or at times assimilation.

cidedly harmful to the field. The Pasteur effect of Pasteur (always sheerly experimental) may, though does not necessarily, operate until the last cleavage product is in air spirited away, by whatsoever experimental means Dame Nature may deign from occasion to occasion to employ, and this quite regardless of how much or how little her means are in theory or design understood by man. All that we can allow Dixon's definition is that it may be called the Pasteur-Dixon definition (implication or restriction) and in this sense it can be very useful and experiment-provoking indeed, even if not entirely general. Conversely to the Dixon view, it is to be said that definition of the Pasteur effect of Pasteur in terms of O_2 suppression of fermentation products formed renders superfluous and redundant definition in terms of O_2 suppression of carbohydrate substrate destruction.

On the Occurrence, Acceleration and Inhibition of Aerobic Fermentation. Perhaps the most important qualitative concept largely missing from Pasteur's writings is that of fermentation under conditions of *adequate aeration,* where diffusion of O_2 is clearly not a limiting factor, in other words, "aerobic fermentation" as it is technically understood today. Pasteur considered, for example, that with adequate aeration yeast would in general not produce alcohol because the rate of oxygen consumption would then be great enough to oxidize away any alcohol formed. He said, thus (108a),

"(In the fully aerobic case) the sugar used up would furnish no alcohol, or at least, if alcohol were formed, it would be decomposed immediately." (p. 133).

"Yeast which lives in the presence of oxygen and can assimilate as much of that gas as is necessary to its perfect nutrition ceases *absolutely* to be a ferment at all." (p. 254) (writer's italics). ".... when its respiratory power becomes null, its fermentative power is at its greatest." (p. 254).

In bacteria and fungi, aerobic (non-alcoholic) fermentations of a sort were recognized to occur in some cases, but the broad modern conception of aerobic fermentation as developed since Meyerhof's work with muscle and yeast, and Warburg's work with tumors, received no emphasis at Pasteur's hands.

But Pasteur actually made no special point of there being no true aerobic fermentation in the modern sense, and so I believe there is no reason, on grounds of consistency, to restrict the Pasteur effect to cases where oxidation predominates over equivalent fermentation ($Q_{O_2} \geqq Q^N_{CO_2}/3$; $Q^{O_2}_{CO_2} = 0$), especially since so much of the main interest in the Pasteur effect has centered, since Meyerhof's Nobel Prize work, on many cases to the contrary ($Q^{O_2}_{CO_2} =$ aerobic fermentation > 0). In Table II is compiled, in this connection, the

TABLE II.   The Occurrence of Aerobic Glycolysis in Nature and in the Laboratory: Normal,
Accelerated and Inhibited

(A Compilation[a])

Type and affecting agent	Tissue or organism	Reference
Normal		
(spontaneous agents)		
	minced muscle, active muscle	Meyerhof (95), etc.
	tumors, benign or malignant, embryo	Warburg (139)
	tissue cultures (fibroblasts, osteoblasts)	Warburg and Kubowitz (138, p. 516); Lipmann (81); Laser (77)
	epithelium, embryonic heart	Warburg and Kubowitz (138, p. 516)
	retina	Warburg, Posener and Negelein (141)
	retina, rat or mouse chorion, chicken allantois	Laser (78, 79)
	white bone marrow cells, testis, embryonic lens, placenta	Fujita (57)
	non-nucleated red blood cells	Negelein (105) (Claude Bernard)
	leucocyte polynuclear exudate, leucocyte monocyte exudate	Fleischmann and Kubowitz (54)
	chick embryo	Krebs (74)
	sperm (human)	MacLeod (91)
	kidney medulla	György, Keller and Brehme (64); Dickens and Weil-Malherbe (39)
	cartilage	Bywaters (21); Dickens-Weil-Malherbe (96)
	tuberculous lymph gland	Dickens and Simer (38).
	Rhizopus fungus	Waksman and Foster (137)
[c] yeasts commonly		Meyerhof (96), etc.
Accelerated[b]		
(Pasteur effect decrease)		
[d] cyanide ester (ethyl isocyanide)	tumor, tissues	Warburg (138)
[d] phenosafranine	brain, tumor	Dickens (32, 33, 34)
[d] phenosafranine	brain, tumor	Baker (4)
[d] phenylhydrazine	tumors	Dickens (30)
[f] HCN	chopped muscle	Meyerhof (94)
[f] HCN	tumor, embryo	Warburg, Posener and Negelein (141)
[f] O_2 deficiency	embryo	Warburg, Posener and Negelein (141)
[f] HCN	non-nucleated red blood cells	Negelein (105)
[d] methemoglobin	nucleated red blood cells	Engelhardt and Shapott (49)
[e] 4-6 dinitro-*o*-creso, dinitrophenol	tumor, kidney	Dodds and Greville (45)
thionine	kidney	Dickens (31)
thionine, prune, methylene blue, cresyl violet, 4-6 dinitro-*o*-cresol, *o*-chlorophenol-indophenol	tumor	Elliott and Baker (47)
[d] 1-amino-2-naphthol-6-sulfonic acid		Krah (73); Eicholtz (48)
[d] glutathione, guanidine, arginine	tumor	Bumm and Appel (17)
[d] glutamate, maleate	tumor	Weil-Malherbe (143)
pyruvate	tumor	Dickens and Greville (36)
boiled tumor extract	muscle	Frisch and Willheim (56)
Ringer's solution	embryo, testis	Negelein (105); Warburg (139)
[e] alkali salts (K, Cs, Rb), or removal of Ca	brain only	Ashford and Dixon (2); Dixon (42); Dickens and Greville (37)

TABLE II.—Continued

Type and affecting agent	Tissue or organism	Reference
Accelerated[b]		
(Pasteur effect decrease)		
[d] low pO_2, CO	retina, chorion, allantois, sarcoma	Laser (78, 79)
[d] butter yellow feeding (rat)	liver	Nakatani, Nakano and Ohara (104); Kinosita (70)
[c] continued growth in HCN	yeast	Pett (109)
[ce] grinding	resting, substrate-free yeast cells	Stier and Stannard (132)
[cd] glutathione	baker's yeast	Quastel and Wheatley (113)
[ce] glutathione, cysteine	yeast	Runnström and Sperber (117, 118)
[c] low pO_2, CO, HCN, azide, when Q_{O_2} decreased 45 p.c. or more	carrot root	Goddard and Marsh (62)
[cd] HCN	algae	Genevois (60)
Inhibited		
(Pasteur effect increase)		
2-6-dichlorophenol-indophenol	muscle extract	Lipmann (82)
[e] pyocyanine and related dyes	testis, tumors	Friedheim (55)
[e] pyocyanine, toluylene blue, Bindschedler's green, methyl Capri blue, ferricyanide	tumor	Dickens (31, 33)
[d] In CO, by light	liver, allantois	Laser (79)
[d] ferricyanide	tumor only	Mendel and Strelitz (92)
l-glyceric aldehyde	tumor, tissues	Mendel, Strelitz and Mundell (92, 93)
lactate	tumor	Dickens and Greville (36)
lactate, fumarate	minced pigeon breast	Greville (63)
dicarboxylic acids, fumarate	minced and sliced tumor	Boyland and Boyland (15)
lactoflavin	tissue cultures	Laser (80)
radiation at low temperature	tumor and tissue	Crabtree (27)
iodoacetate	tumor	Crabtree and Cramer (28)
[cf] iodoacetate	yeast	Lundsgaard (90)
[c] naptholsulfonate-indophenol	yeast maceration juice	Lipmann (82)
[ce] pyocyanine, thionine, methylene blue, gallophenine, indigosulfonate (all irreversibly, with enzyme destruction)	yeast extract	Michaelis and Smythe (101)
[c] 1-napthol-2-sulfonate indophenole, brilliant alizarine blue,[e] rosinduline GG[d]	yeast extract	Michaelis and Smythe (101)
[c] yeast water	baker's yeast	Meyerhof and Iwasaki (98)

[a] Not including bacterial fermentation.

[b] Apart from obvious mechanical (grinding, freezing, thawing) or thermal harm or asphyxiation.

[c] Alcoholic fermentation, not glycolysis (lactic acid fermentation).

[d] Q_{O_2} essentially unaffected at concentration affecting aerobic fermentation.

[e] Q_{O_2} increased (or established above zero).

[f] Q_{O_2} decreased.

great majority of cases of spontaneous aerobic glycolysis, together with accelerating and inhibiting agents, known or claimed at present. As mentioned in discussing Table I, one might have increased the size of the latter by further drawing upon Table II.

It will be seen that a great deal of the aerobic fermentation, spontaneous, accelerated or inhibited, is to be found in tumor material, and to this we shall return after the following section on the measurement of the anaerobic and aerobic glycolysis in terms of oxidation quotients, to which we may now turn.

The Quantitative Measurement of the Pasteur Effect: Oxidation Quotients. The most direct measurement of the Pasteur effect is evidently given by the aerobic inhibition of

fermentation, thus (on an absolute basis) $Q^{N_2}_{CO_2} - Q^{O_2}_{CO_2}$, or (on a percentage basis) $100(Q^{N_2}_{CO_2} - Q^{O_2}_{CO_2})/Q^{N_2}_{CO_2}$. Under the influence of the view that respiration in particular rather than oxygen gas in general causes the aerobic decrease in fermentation, Warburg earlier believed that the Pasteur effect was measured better by what he called (139, pp. 124, 241) the "Meyerhof quotient",

$$\frac{\text{anaerobic fermentation} - \text{aerobic fermentation}}{\text{respiration}} =$$

$$\frac{Q^{N_2}_{CO_2} - Q^{O_2}_{CO_2}}{Q_{O_2}} \qquad (2)$$

a quotient numerically equal to one third the "Meyerhof oxidation quotient" (so-named for purposes of distinction, 20, p. 132) of Equation 1, since the denominator in the latter is divided by 3 to express the oxygen consumption in terms of equivalent (3-carbon) cleavage product. Both quotients are purely experimental expressions independent of any theory, and in particular of the Meyerhof cycle theory, as specifically stated by Warburg early in his work (139, p. 242).[5] The former quotient commonly varies numerically from 1 to 2, and the latter from 3 to 6, in cases where fermentation is considerably decreased aerobically and where Q_{O_2} by no means predominates over $Q^{N_2}_{CO_2}$.

[5] One has heard so often during the past twenty years, following Meyerhof, that the Meyerhof quotient is a proof of the Meyerhof cycle; but the latter is obviously only one of many theories or explanations of the former, as has become especially evident during the past two or three years. I for one would be greatly relieved never to hear nor to feel called upon to rise to correct statements implicating a proof of cycle by quotient, but this is probably almost too much to hope for, so strongly has the impression gained ground in the less critical sources such as texts. The great significance of the oxidation quotients and Meyerhof's experimental work in connection therewith is that they show that cleavage product disappearance may be several times greater than can be accounted for by oxidation; a finding well meriting its Nobel prize, especially when one pauses to consider how much further along the metabolic field could have been had this become known and established some fifty years earlier than 1920. But there is no use lamenting not spilt but delayed milk, especially now these twenty years after its eventual arrival. And yet it seems to have been a rather simple matter; reminiscent, to my mind, of the missing of the infra red and ultraviolet regions of the spectrum by Newton. "This great man" as he was referred to in the Philosophical Transactions for over two centuries, studied the visible spectrum literally backwards and forwards about 1670, yet it was not until 1800 that Sir William Herschel had the common sense to put a thermometer beyond the red end of the spectrum and note a considerable rise in temperature; and upon publication of

this was brought forth early the next year the simultaneous discovery of the ultraviolet region by Ritter in Germany and Wollaston in England within a few days of each other.

The oxidation quotient bears much the same relation to the earlier metabolic work predicating adequate oxidation of cleavage product as the second law of thermodynamics does to the first law. Anyone can readily grasp the meaning of the first law now, but the subtleties of the second law take some time to see, and so with the oxidation quotients. This suggests, in the colloquial vein of this paper, a rather striking difference in the method of operation between Meyerhof and Warburg, and is rewarding to comment upon it in connection with its effects upon the metabolic field as a whole. Meyerhof, a pioneer, goes after conceptions and theories and principles and complications of reactions which are undeniably subtle and difficult of general appreciation; Warburg, on the other hand, a classicist, produces largely what might be called concrete facts and laws and apprehends (isolates) and determines identifiable materials and chemicals. In the middle of the last century, as Americans, Meyerhof would have followed the advice of Greeley and "gone West", Warburg would have stayed back in Boston; in 1620 Meyerhof would have been on the Mayflower, Warburg at an *Akademie* on the Continent. The one plunges ahead, making sizable mistakes for every real but indispensable advance; the other stays behind, in territory where in a sense no mistakes need be made, and consolidates, purifies, and redistills until nothing more need be done: a Park Avenue pioneer maybe, for those who insist that he *is* a pioneer, but a sheltered one indeed. On the whole it is work of the type of Meyerhof's rough-hewn variety that is the more stimulating and irritating to other workers. For twenty years Meyerhof has kept every biochemist humming more or less, but Warburg's work is so perfectly carried out and in general so easily and readily understood that for a long time afterward, even a decade, it inspires mainly admiration and acceptance but little urge to further advance; this was true of his work on photosynthesis, and then with cancer and the *Atmungsferment*.

This type of quotient has undergone considerable refinement, change in mode of expression, and actual modification in order to fit special experimental conditions and measurement requirements. Thus Dixon (41) has expressed it in terms of C_6 compound substrate instead of C_3 cleavage product, an experiment-provoking procedure in particular cases. Equation 3 is a form used by Meyerhof and many others later for cases in which anaerobic and aerobic fermentations are not compared, but purely aerobic cases in which cleavage product disappearance is measured (here generally but not necessarily in the absence of fermentable substrate). In these cases, not the total oxygen consumption but the extra oxygen consumption (increase over control with no added cleavage product) is employed in the quotient, which may be termed the

Meyerhof aerobic oxidation quotient

$$= \frac{\text{total cleavage product disappearing}}{\text{equivalent "extra" oxygen consumption}}. \qquad (3)$$

The same quotient, for the purely aerobic case, but on a basis of *total* oxygen consumption, may be called the

Kluyver oxidation quotient

$$= \frac{\text{total cleavage product disappearing}}{\text{equivalent total oxygen consumption}} \quad (4)$$

after Kluyver, who used it in 1931 (71) to throw some doubt on the use of the previous one (Equation 3) as support for the Meyerhof cycle theory:

$$(5)$$
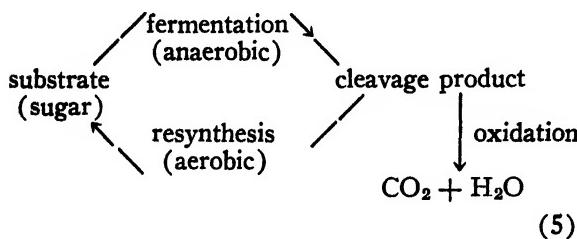
The argument, which cannot be repeated here, was considerably elaborated by the writer upon the basis of the data of Tables III and IV. [cf. (20), Tables IX and X], in which tables the Meyerhof aerobic oxidation quotient and the Kluyver oxidation quotient and their methods of calculation from experimental data are illustrated. In Table IV, the lactate disappearance, not having been measured directly (chemically), is calculated as equal to the measured "carbohydrate synthesized" (Col. 7) plus the lactate oxidized as measured for quotients 3 (Col. 10) and 4 (Col. 11) by, respectively, the extra and the total (equivalent) oxygen consumptions. The two quotients 3 and 4 represent the two extremes of uncertainty introduced by not knowing whether the lactate oxidized is accounted for by the extra or the total oxygen consumption or intermediate values. Even here it is assumed that the lactate oxidized is at least as great as the extra oxygen consumption, but, as we shall see shortly, there are cases where lactate stimulates oxygen consumption without itself being burned correspondingly, *i.e.*, where low respiratory quotients are involved and are

TABLE III

Directly Measured Oxygen Consumption, Lactic Acid (L.A.) Disappearance, and Carbohydrate (Cbh: Glycogen, etc.) Synthesis, in mg./gm. Gastrocnemius Muscle, during Recovery [a] after Prolonged Stimulation [b] (Meyerhof, 94, p. 24-5).

Exp. No.	O_2 consumed			Total L.A. disappeared	L.A. equivalent of total O_2	Net L.A. resynthesized (calc.)	Net Cbh synthesized	Total L.A. disappeared / Equivalent extra O_2 (Meyerhof aerobic oxidation quotient)	Total L.A. disappeared / Equivalent total O_2 (Kluyver oxidation quotient)
	Total	Resting unstimulated	Extra (control)						
1	2	3	4	5	6	7	8	9	10
calc.			2-3		2 x (90/96)[c]	5-6		(5/4) x (96/90)[c]	5/6
9	1.48	0.64	0.84	2.36	1.39	0.97	0.96	3.0	1.7
10	1.22	0.54	0.68	2.12	1.14	0.98	1.09	3.5	1.9
11	1.45	0.72	0.73	(2.8)	1.36	1.44	1.57	4.1	2.1
12	1.50	0.79	0.71	2.5	1.41	1.09	0.81[d]	3.8	1.8
13	1.35	0.57	0.78	(2.8)	1.26	1.54	1.38[d]	3.8	2.2
14	1.35	0.60	0.75	(2.8)	1.26	1.54	1.28[d]	4.0	2.2
Av.	1.39	0.64	0.75	2.56	1.30	1.26	1.18	3.5	2.0

[a] 21-23.5 hr. (average, 22.5).
[b] 20-30 min.
[c] M.W.L.A. (90)/M.W. 30₂(96).
[d] Glycogen only.

TABLE IV

Directly Measured Oxygen Consumption and Carbohydrate (Cbh) Synthesis (?), but not Lactate (L.A.) Disappearance, in mg./Gastrocnemius Muscle, with Added Lactate (Meyerhof, Lohmann and Meier, Biochem. Z. *157*, 468, 1925).

Exp. No.	Muscle wt. (gm.)	Time (hr.)	O_2 consumed			Cbh "synthesized"[a] $(+ L.A.) - (- L.A.)$	L.A. equivalent of extra O_2	Total L.A. disappearing (calc.)	Total L.A. disappeared Equivalent extra O_2 (Meyerhof aerobic oxidation quotient)	
			+ L.A.	− L.A.	extra					
1	2	3	4	5	6	7	8	9	10	11
					4-5		6 x (90/96)	7 + 8	9/8	7 + 4(90/96)/ 4(90/96)
1	1.30	21	1.98	1.35	0.63	2.1	0.59	2.7	4.6	2.1
2	1.30	18.5	1.46	1.12	0.34	2.0	0.32	2.3	7.2	2.4
3	2.08	18.5	2.41	1.73	0.68	1.6	0.64	2.2	3.4	1.7
4	1.49	6.5	1.71	0.73	0.98	1.1	0.92	2.0	2.2	1.7
5	1.28	16	1.39	0.73	0.66	1.7	0.62	2.3	3.7	2.3
6	1.05	15.5	0.89	0.68	0.21	0.95[b]	0.20	1.15	5.7	2.1
7	1.50	15.5	1.62	1.12	0.50	1.05[b]	0.47	1.5	3.2	1.7
Av.	1.43	16	1.64	1.07	0.57	1.5	0.54	2.0	4.3	2.0
3[c]	0.19	5.5	0.10	0.04	0.06	0.32	0.06	0.8	6.2	4.4

[a] Carbohydrate decrease in control with no lactate, compared to lactate present (actually net carbohydrate not lost, since carbohydrate in + L.A. did not alter appreciably).
[b] Glycogen only.　[c] Frog sartorius (Meyerhof and Lohmann, Biochem. Z. *171*, 430, 1926).

not correspondingly raised along with the increased oxygen consumption caused by the lactate.

As a further refinement, the numerator of quotients 3 and 4 is often further corrected, especially in the work of the Shorr school, for any disappearance of small amounts of residual cleavage product in the controls, which is generally concerned in lactate disappearance in many tissues, where it is almost impossible to obtain zero lactate concentrations. Thus quotient 3 takes the form (125)

$$\frac{\text{lactate disappearance in lactate} - \text{lactate disappearance in non-nutrient medium}}{\text{lactate equivalent of extra oxygen consumption in lactate}} \quad (3')$$

For the Meyerhof oxidation quotient (Equation 1) the Shorr group write, perhaps a little cryptically, but with no change in meaning,

A further and really significant modification of the various quotients has been proposed by Shorr (135) to take care of situations where respiratory quotients (CO_2 produced/O_2 consumed) are less than unity. Work with all of the foregoing oxidation quotients has largely involved measured or assumed R.Q. values of about unity. As is quite clear from Tables V and VI, which present not only further data on the meaning and calculation of some of the foregoing quotients, but also a concise summary of a good deal of the pertinent work of the Shorr group on normal, depancreatized, Houssay and incubated tissue, the R.Q. can often be well below unity in a meaningful

$$\frac{\text{lactate appearing in } N_2 + \text{lactate which would have disappeared in } O_2}{\text{lactate equivalent of total oxygen consumption}} \quad (4')$$

TABLE V. Comparison of the Respiratory Metabolism and Lactic Acid Oxidative Quotient of Excised Cardiac or Skeletal Muscle from Normal, Depancreatized and Houssay Dogs and Incubated Surviving Tissues*

Type of Animal and Tissue	Number of experiments	Data from Shorr (135) Table number	Respiratory Quotient h			Oxygen Consumption (cc./gm./hr.)			Total fermentable carbohydrate + lactate (mg./gm./hr.)	Net lactate disappeared. M/80 lactate-non-nutrient. (mg./gm./hr.)	Anaerobic Glycolysis (mg./hr.)		Oxidation Quotient 1			
			Non-nutrient	M/80 lactate, inc.	0.2 p.c. glucose, inc.	Non-nutrient	M/80 lactate, inc.	0.2 p.c. glucose, inc.			Non-nutrient	0.2 p.c. glucose.	Meyerhof	Meyerhof-Shorr	Kluyver	Kluyver-Shorr
Column No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Cardiac																
Normal f	9	5	0.84	0.95(0.11)	0.97(0.13)	0.58	0.91(0.33)	0.53(−0.05)	7.9d	1.06a	0.66a	2.6a	3.5	4.2	1.2	1.4
Normal f		(c)	0.81		0.89(0.08)	0.47		0.54(+0.07)								
Houssay f	4	1	0.88	0.95(0.07)	0.95(0.07)	0.34	0.60(0.26)	0.34(0.00)	9.0d	1.05a	0.48a	2.3a	4.4	5.1	1.8	2.1
Depancreatized g	8	8	0.79	0.80(0.01)		0.86	1.15(0.29)	0.68(0.05)	13.5d	1.07a	1.03a	2.1a	4.0	12	0.9	2.8
Depancreatized g	7	9(b)	0.75		0.73(−0.02)	0.63										
Incubated e "	5	(c)	0.85		0.99	0.59		0.53								
Incubated Normal		(c)	0.89		1.00	0.57		0.86								
Skeletal																
Normal f	7	(b)	0.94	0.91(−0.03)		0.23	0.33(0.10)		1.3a	0.55a	1.1	1.7	5.9	8.4	1.7	2.4
Houssay f	7	10	0.80	0.86(0.06)		0.39	0.51(0.12)		0.9a	0.6a	0.44a	0.41a	5.4	10	1.2	2.2
Depancreatized g	5	6	0.73	0.78(0.05)		0.25	0.29(0.04)		0.66a	0.04a	0.38	0.36	1.0	4.2	0.2	0.5

* All oxidative quotients based on chemically determined lactic acid; all (gm.) muscle weights moist.
a Unpublished data of Shorr, Loebel, Richardson, Sweet and Malam, kindly supplied by Dr. Shorr.
b Ref. 126; c, Ref. 122; d, Ref. 135, Table XII.
e Incubated 10 hours in Ringer-phosphate-glucose at 37.5° C.
f 16 hours post-absorptive.
g Fasted several days.
h R.Q. in pyruvate (cardiac): normal and Houssay, 1.35; depancreatized and theoretical, 1.2.
1 See footnotes b, c, d, e, f, Table VI.

TABLE VI. The Respiratory Metabolism and Lactic Acid Oxidative Quotient of Skeletal Muscle Strips of the Normal (Fed and Fasted) Dog (*cf.* Shorr, 135, Table IV) Arranged in Order of Decreasing Respiratory Quotient.

Experimental Condition	Respiratory Quotient			Oxygen Consumption/2 hrs./gm.			Net lactate disappearance [a]	Oxidation Quotient [b]			
	Non-nutrient	Lactate M/80	Increase	Non-nutrient	Lactate M/80	Increase		Meyerhof [c]	Meyerhof-Shorr [d]	Kluyver [e]	Kluyver-Shorr [f]
Column No.	1	2	3	4	5	6	7	8	9	10	11
				cc. mg.	cc. mg.	cc. mg. p.c.					
Normal Fed	1.01	0.96	−0.05	0.46 0.62	0.66 0.88	0.20 0.27 +44	0.90	3.6	4.1	1.0	1.2
Normal Fed	1.00	—	—	0.57 0.76	0.99 1.32	0.42 0.56 +74	1.33	2.6	—	1.0	—
Normal Fed	0.96	0.92	−0.04	0.38 0.51	0.57 0.76	0.19 0.25 +50	0.50	2.2	2.9	0.7	0.9
Normal Fed	—	0.88	—	0.43 0.57	0.60 0.80	0.17 0.23 +40	0.90	4.2	7.0	1.1	1.8
Normal Fed	0.85	0.85	±0	0.48 0.64	0.65 0.87	0.17 0.23 +36	1.50	7.0	14	1.7	3.4
16 day fast	0.82	0.76	−0.06	0.47 0.63	0.51 0.68	0.04 0.05 + 9	0.70	14	70	1.0	5.1
46 day fast	0.79	0.82	+0.03	0.48 0.64	0.58 0.78	0.10 0.13 +21	1.12	9.2	22	1.4	3.6
14 day fast	0.77	0.79	+0.02	0.47 0.63	0.56 0.75	0.09 0.12 +19	2.0	18	59	2.7	9.0
54 day fast	0.72	0.73	+0.01	0.44 0.59	0.47 0.63	0.03 0.04 + 7	0.72	19	194	1.1	11.0

[a] Derived from the aerobic chemically measured disappearance of lactate corrected for disappearance in control with no lactate.

[b] Thermodynamic theoretical maximum Meyerhof oxidative quotient [based upon lactate synthesis to higher carbohydrate, and lactate or carbohydrate oxidation (R.Q. = 1)] = 10-12; [based on protein or fat oxidation (R.Q. = 0.7-0.85) = 22-25] (*cf.* Burk, 19).

[c] (96/90) (Col. 7/Col. 3), where (96/90) = m.w. 30/m.w. L.A.).

[d] (96/90) (Col. 8)/Shorr R.Q. factor, where Shorr R. Q. factor = (obs. R.Q. in lactate - 0.70)/(1.00 - 0.70).

[e] (Col. 7/Col. 2) × 1.08, where 1.08 represents average factor converting net lactate disappearance to actual lactate disappearance in M/80 lactate.

[f] (Col. 10)/(1.08 × Shorr R.Q. factor).

way, and indeed as low as about 0.7 corresponding to fat oxidation. Since the interest heretofore has presumed carbohydrate (or carbohydrate equivalent) oxidation, Shorr (135) now introduces a factor aimed at retaining this interest and at the same time clarifying the issue in regard to non-carbohydrate oxidation certainly involved. Shorr multiplies the quotient denominators (or in effect divides the quotients) by the term

$$\text{Shorr factor} = (\text{observed R.Q. in lactate} - 0.70)/(1.00 - 0.70), \quad (6)$$

employed earlier by Dickens and Simer (38, p. 1316) as "% of total O_2 for carbohydrate." It is evident that the Shorr factor is aimed at determining how much of the observed oxygen consumption can be regarded as "average carbohydrate oxidation," since the factor is a measure of the decrease in observed R.Q. from unity (factor numerator) in relation to the total possible decrease of 0.30 at pure fat oxidation and no carbohydrate (or lactate) oxidation (factor denominator). With the observed R.Q. values being less than unity the effect of the factor is obviously to raise the value of the calculated oxidation quotient. As is evident from the tables, the increase in quotient becomes many fold as the R.Q. approaches 0.70, and this is true even if very considerable allowance is made for the much greater magnification errors of the calculated values involved.

There are evidently perfectly valid experimental quotient values above 10 or 12, corresponding to more than the theoretical thermodynamically calculated maximum possible values based on carbohydrate oxidation (or lactate oxidation) and lactate synthesis to higher carbohydrate. Values up to perhaps 25 are permitted upon the basis of pure fat oxidation, and of course still higher values if lactate is not synthesized all the way to higher carbohydrate but to, say, triose or only pyruvic acid. These maximum thermodynamic expectations must be reduced, of course, by whatever efficiency factors may be involved (25 to 100 p.c.). The experimentally obtained oxidation quotients obviously indicate, quite independently of any direct answer given by the respiratory quotients, non-carbohydrate (or non-lactate) oxidation of varying degree. This holds for both the total oxygen consumption and the extra oxygen consumption stimulated by lactate, and is especially true with the fasted animals in Table VI. One of the most remarkable points, indeed, of the many points established by the data of Tables V and VI, is the large rise (e.g. in depancreatized dog cardiac muscle) in oxygen consumption and oxidation quotient without a corresponding rise in the low R.Q. values, i.e., a non-specific extra oxygen consumption caused, but not accounted for, by

the added lactate. The oxidative quotient here, large in itself, shows a marked Shorr correction factor, and the anaerobic glycolysis is considerably increased over the normal even though the net aerobic lactate disappearance is essentially unchanged.

In various instances, on the other hand, the extra oxygen consumption rises correspondingly with the R.Q., upon addition of lactate, and there is then no special reason to assume other than equivalent carbohydrate oxidation in this rise, or indeed to employ a Shorr correction factor to the Meyerhof oxidation quotient (though still reason to use the Kluyver quotient). Nevertheless, one still runs, even here, into the moot Kluyver-Burk question as to just where the lactate is disappearing in the cases of large oxidation quotients, i.e., how much into oxidation, into resynthesis in the Meyerhof sense, or into general synthesis (20, pp. 151-5)? It is seen that the Kluyver oxidation quotients of Tables V and VI, being close to unity or less, tend to make little of possible resynthesis, as compared with the considerably higher Meyerhof oxidation quotients, whereas the Kluyver-Shorr quotients are more suggestive in this connection, and very possibly more pertinent, at least with the cases of low R.Q. values as in fasted dogs. The significance of the various oxidation quotients given in Tables V and VI clearly remains open for much further investigation, especially on a comparative basis. Certainly one of the most concrete and readily understandable quotients is that of the diabetic skeletal muscle, where addition of lactate causes almost no increase in R.Q. or Q_{O_2} or actual lactate disappearance, and the Meyerhof oxidation quotient is unity; corrected for the Shorr factor it appears to be significantly greater than unity even if the transformations going on are almost negligible so far as lactate is concerned.

Tables V and VI concern many matters which can scarcely be touched upon here, e.g., general metabolic similarities between normal and Houssay as compared with diabetic surviving tissues, with the implicated hormonal interplay and antagonism brought out especially by the Houssay animal work; comparisons between results with surviving tissues and whole animals or organs; far-reaching differences between cardiac and skeletal muscle in relation to glycogen synthesis and storage, total fermentable carbohydrate and metabolic functions; and the marked changes in incubated surviving tissue involving raising of the R. Q. to unity (especially in the diabetic tissue) with oxygen consumption maintaining itself practically constant. For these matters reference must be made to the summarizing paper of Shorr (135), which represents a remarkably extensive and profoundly interesting set of data and discus-

TABLE VII. Quantitative Classification of Freshly Isolated Animal Tissues According to Respiration, Aerobic Glycolysis, Anaerobic Glycolysis and Derived Relations, Including U, the Fermentation-Excess for Maximum Pasteur Reaction Activity* (Warburg, 138, 139, cf. 20).

Tissue	I Respiration Q_{O_2}	II Aerobic glycolysis† $Q^{O_2}_{CO_2}$	III Anaerobic glycolysis $Q^{N_2}_{CO_2}$	IV Glycolysis inhibition (absolute Pasteur effect) $Q^{N_2}_{CO_2}-Q^{O_2}_{CO_2}$	V Percent inhibition of glycolysis by air (p.c. Pasteur effect) $100(Q^{N_2}_{CO_2}-Q^{O_2}_{CO_2})/Q^{N_2}_{CO_2}$	VI Meyerhof oxidation quotient $\dfrac{Q^{N_2}_{CO_2}-Q^{O_2}_{CO_2}}{Q_{O_2}/3}$	VII Anaerobic glycolysis Equivalent respiration $\dfrac{Q^{N_2}_{CO_2}}{Q_{O_2}/3}$	VIII Aerobic glycolysis Equivalent respiration $\dfrac{Q^{O_2}_{CO_2}}{Q_{O_2}/3}$	IX Fermentation-Excess, U $Q^{N_2}_{CO_2}-2Q_{O_2}$	X Aerobic Glycolysis Fermentation Excess $Q^{N_2}_{CO_2}-2Q_{O_2}$
Stable										
Muscle fascia (rat)	(trace)	(trace)	(trace)	(trace)						—
Kidney (rat)	21	0	3	3	100	0.4	0.5	0	−39	—
Thyroid gland (rat)	13	0	2	2	100	0.5	0.4	0	−24	—
Liver (rat)	12	0.6(0)	3	2.6(3)	80(100)	0.6(0.7)	0.7	0.2(0)	−21	—
Intestinal mucous membrane (rat)	12	1.6(0)	4	2.4(4)	60(100)	0.6(1.0)	1.0	0.4(0)	−20	—
Spleen (rat)‡	12	2	8	6	75	1.5	2.0	0.5	−16	—
Testis (rat)	12	7(0)	8	1(8)	12(100)	0.2(2.0)	2.0	1.7(0)	−16	—
Pancreas (rabbit)	5	0	3	3	100	2.0	1.8	0	−7	—
Pancreas (dog)	3	0	4	4	100	4.0	4.0	0	−2	—
Submaxillary (rabbit)	4	0	3	3	100	2.2	2.1	0	−5	—
Lymph gland (man)	4	2	5	3	60	2.2	3.7	1.5	−3	—
Thymus (rat)	6	0.6	8	7.4	93	3.6	4.0	0.3	−4	—
Brain cortex (rat)	11	2.5	19	16.5	87	4.5	5.1	0.7	−3	—
Normal tonsil (man)	6	3	12	9	75	4.5	6.0	1.5	0	0
Hyperplastic tonsil (man)	9	10	18	8	44	2.7	6.0	3.3	0	0
Growing										
Placenta (rat)‡	7	10	14	4	28	1.7	6.0	4.2	0	0
Embryo										
(rat, 3 mg. in serum)	12	0(7)	13	13(6)	100(46)	3.3(1.5)	3.3	0(1.8)	−11	—
(rat, 0.9 mg. in serum)	13	6	23	17	74	3.9	6.3	1.4	−3	—
(rat, 0.5 mg. in serum)	(14)	15(0)	28	13(28)	46(100)	2.8(6)	6.0	3.3(0)	0	0
Embryo										
(chicken, 1.7 mg.)	10	1	20	19	95	5.7	6.0	0.3	0	0
(chicken, after 2 hr. O₂-lack)	7	7	20	13	65	5.5	8.6	3.0	+6	+30 p.c.
(chicken, in M/1000 HCN)	4	12	20	8	40	6.0	15.0	9.0	+12	+60 p.c.

Non-malignant										
Bladder papilloma (man)	13	16	26	10	39	2.3	6.4	3.7	0	—
Nasal polypus (man)	5	5	14	9	64	5.4	8.4	3.0	+4	+27 p.c.
Malignant										
Bladder carcinoma (man)	10	24	36	12	33	3.6	10.8	7.2	+16	+45 p.c.
Flexner-Jobling sarcoma (rat)	7	25	31	6	20	2.6	13.0	10	+17	+55 p.c.
Jensen sarcoma (rat)	9	17	34	17	33	5.6	11.3	5.6	+16	+47 p.c.
Rous sarcoma (chicken)	5	20	30	10	33	6.0	18.0	12.0	+20	+67 p.c.
Groin-cell sarcoma (man)	5	16	28	12	43	7.2	16.8	9.6	+18	+64 p.c.
Special (?)										
Retina (rat)	31	45	88	43	46	4.2	8.5	4.3	+26	+30 p.c.
Malignant, mixed (carcinoma in man)										
Skin (50 p.c. tumor)	7.0	11.9	16.8	4.9	29	2.1	7.2	5.1	+3	+18 p.c.
Penis (35 p.c. tumor)	5.9	11.9	14.8	2.9	20	1.5	7.5	6.0	+3	+20 p.c.
Larynx (80 p.c. tumor)	7.5	14.6	19.3	4.7	24	1.9	7.7	6.0	+4	+22 p.c.
Mucous membrane, lower jaw (50 p.c. tumor)	4.8	10.1	18.2	8.1	45	5.1	10.1	6.3	+9	+47 p.c.
Colon (? p.c. tumor)	7.5	17.4	26.7	9.3	35	3.6	10.7	7.2	+12	+44 p.c.
Nose, lupus carcinoma (50 p.c. tumor)	3.6	14.6	19.4	4.8	25	3.9	17.2	12.3	+12	+63 p.c.
Penis (50 p.c. tumor)	7.9	18.8	28.6	9.8	34	3.6	10.8	7.2	+13	+45 p.c.
Skin, external ear (67 p.c. tumor)	3.5	15.8	20.9	5.1	24	4.5	17.9	13.5	+13	+63 p.c.
Penis (60 p.c. tumor)	2.0	11.5	18.3	6.8	37	10.2	27.5	17.7	+14	+78 p.c.
Larynx (? p.c. tumor)	3.3	10.8	21.2	10.4	49	9.3	19.2	9.6	+15	+70 p.c.
Face skin, lupus carcinoma (30 p.c. tumor)	3.5	12.9	22.4	9.5	42	8.1	19.2	11.1	+16	+69 p.c.
Rectum (? p.c. tumor)	5.2	15.6	27.2	11.6	43	6.6	15.7	9.0	+17	+62 p.c.
(In tumor serum, not Ringer's solution)										
Mammary gland (20-40 p.c. tumor)	2.6						13.8		+6	+53 p.c.
Skin (50-70 p.c. tumor)	3.1						13.5		+8	+56 p.c.
Lip (50-70 p.c. tumor)	3.4						14.4		+10	+58 p.c.
Liver metastasis of gall bladder carcinoma (10-20 p.c. tumor)	1.1		13.6				37.1		+11	+84 p.c.

* Usual conditions, about 37° C., pH 7.7, 0.2 p.c. glucose, 2.5 × 10⁻² M NaHCO₃, Ringer's solution.

† Parenthetical values for serum or Ringer's solution where other respective values are supplied and are different.

‡ Murphy and Hawkins, J. Gen. Physiol. 8, 115 (1925).

Q_{O_2} = mm.³ O₂ respired/mg. tissue dry weight/hr.; $Q^N_{CO_2}$ = mm.³ lactic acid produced aerobically/mg. tissue dry weight/hr.; $Q^{Ns}_{CO_2}$ = mm.³ lactic acid produced anaerobically/mg. tissue dry weight/hr.; (1 mm.³ lactic acid is equivalent to 1 mg.-molecule acid/22,400 or 0.004 mg. lactic acid, so that $Q^{Ns}_{CO_2}$ = 25 means the production of lactic acid corresponding to 10 p.c. of the tissue dry weight per hr.); $U = Q^{Ns}_{CO_2} - 2Q_{O_2}$.

TABLE VIII. Some Post-Warburg Tumor Metabolism Data[a], Especially of Mouse

Tumors	Animal	No. of expts.	I Respiration Q_{O_2}	II Aerobic glycolysis $Q^{O_2}_{CO_2}$	III Anaerobic glycolysis $Q^N_{CO_2}$	IV Glycolysis inhibition (absolute Pasteur effect) $Q^N_{CO_2} - Q^{O_2}_{CO_2}$	V Percent inhibition of glycolysis by air (p.c. Pasteur effect) $100(Q^N_{CO_2} - Q^{O_2}_{CO_2})/Q^N_{CO_2}$	VI Meyerhof oxidation quotient $\dfrac{Q^N_{CO_2} - Q^{O_2}_{CO_2}}{Q_{O_2}/3}$	VII Anaerobic glycolysis Equivalent respiration $\dfrac{Q^N_{CO_2}}{Q_{O_2}/3}$	VIII Aerobic glycolysis Equivalent respiration $\dfrac{Q^{O_2}_{CO_2}}{Q_{O_2}/3}$	IX Fermentation- Excess, U $Q^N_{CO_2} - 2Q_{O_2}$	X Fermentation Excess Aerobic Glycolysis $\dfrac{Q^N_{CO_2} - 2Q_{O_2}}{Q^N_{CO_2}}$
Warburg-cited (grand averages (139a, v.))												
Warburg, 1924												
Flexner-Jobling carcinoma	rat	11	8	24.6	34.8	10.2	29	3.8	13.1	9.2	18.8	+54 p.c.
Human tumors (8)	human	13	5.3	13.3	20.4	7.1	35	4.0	11.5	7.5	9.8	+48 p.c.
Murphy and Hawkins, 1925												
Flexner-Jobling	rat	11	8.4	19.9	29.0	9.1	31	4.3	10.3	7.1	12.2	+42 p.c.
Transplanted tumor I	chick	4	7.5	21.2	27.5	6.3	23	3.4	11.0	8.5	12.5	+45 p.c.
Transplanted tumor IX	chick	17	9.1	14.6	23.5	8.9	38	2.9	7.6	4.8	−5.3	−22 p.c.
Spontaneous tumors	mouse	35	13.6	7.5	25.3	17.8	70	3.9	5.6	1.6	−1.9	−7 p.c.
Crabtree, 1929												
Crocker sarcoma	mouse	13	15.8	16.8	27.8	11.0	39	2.1	5.2	3.2	−3.8	−14 p.c.
Tar carcinoma	"	6	19.8	15.3	24.6	9.3	38	1.4	3.7	2.3	−15	−6 p.c.
37 sarcoma	"	6	15.1	11.8	28.2	16.4	58	3.2	5.6	2.3	−4.0	−14 p.c.
Tar sarcoma Bonné	"	3	13.3	10.0	22.3	12.3	55	2.8	5.0	2.3	−4.3	−35 p.c.
Tar sarcinoma 173	"	8	14.8	15.2	30.2	15.0	50	3.0	6.2	3.1	−0.4	−1 p.c.
Sarcoma 2529	"	6	14.3	15.8	32.2	16.4	51	3.4	6.7	3.3	3.6	+11 p.c.
Sarcoma 2529	"	2	12.0	15.0	29.5	14.5	49	3.5	7.5	3.7	5.5	+19 p.c.
Glycogen-carcinoma 113	"	4	12.0	4.7	14.0	9.3	66	2.2	3.5	1.2	−10	−71 p.c.
Melanotic sarcoma	"	4	8.7	5.5	15.7	10.2	65	3.5	5.4	2.1	−1.3	−8 p.c.

Dickens and Simer, 1930-1 (38)										
Sarcoma 37S	mouse	6.5	19.9	26.2	6.3	24	2.9	12.1	9.2	13.2	+50 p.c.
Tar carcinoma 2146	"	6.9	13.4	22.5	9.1	40	3.0	9.8	5.8	8.7	+39 p.c.
Spindle-cell tar tumor 173	"	3.4	11.0	21.0	10.0	48	8.8	19.1	9.7	14.2	+68 p.c.
Spontaneous carcinoma I	"	7.5	8.1	20.1	12.0	60	4.8	8.0	3.2	5.1	+25 p.c.
Spontaneous carcinoma II	"	11.3	8.8	16.0	7.2	45	1.9	4.2	2.3	-6.6	-41 p.c.
Breast carcinoma SS 371[b]	man	1.7	2.9	7.1	4.2	59	7.4	12.5	5.1	3.7	+52 p.c.
Papillary bladder carcinoma, SS 365[b]	"	1.5	3.1	3.4	0.3	9	0.6	6.8	6.2	0.4	+12 p.c.
Kinosita-cited, 1937 (70)											
3:4-benzpyrene sarcoma	rat	9.3	8.6	24.5	15.9	65	5.1	7.9	2.8	5.9	+24 p.c.
Maruya sarcoma	rat	19.7	22.3	43.3	21.0	48	3.2	13.0	3.4	3.9	+9 p.c.
Jensen carcinoma (Okamoto)	rat	10.0	21.5	32.2	10.7	33	3.8	9.6	6.4	12.2	+38 p.c.
Tar carcinoma (Ebina)	(rat)	14.1	13.7	29.2	15.5	53	3.3	6.2	2.9	1.0	+3 p.c.
(Pathologic)											
Tuberculous lymphatic gland	man	5.5	8.2	14.2	6.0	42	3.3	7.7	4.5	3.2	+23 p.c.

[a] Usually Ringer-glucose-bicarbonate medium. [b] Admixed with normal tissue.

sion, the result of a decade of intensive investigation, and a vigorous tribute to the Lusk school whence it sprang.

The Pasteur Effect in Tumor Metabolism. Tumors provide one of the best types of biological material wherewith to study the Pasteur effect, because of their large capacity for fermentation both aerobically as well as anaerobically. In addition, and partly because of the profound inherent interest in cancer, a great deal of data has been obtained by a large number of workers, so that it is possible to draw conclusions of broad generality. In Tables VII and VIII are gathered together a considerable fraction of the rather more classic Warburg and post-Warburg data of ten to fifteen years ago. These data together with those of Table IX represent, I should say, the first and as yet only peak attained in the search for a specific biochemistry of cancer, and even here the characterization is a quantitative one rather than qualitative. By and large, there is only one established differentiation between the biochemistry of malignant tumors and other tissues. This differentiation is not one of chemical composition but of metabolism, and was established mainly by Warburg in Germany and Dickens in England. As we shall see, the contribution of Dickens was overshadowed by that of Warburg, and has not received the attention it merits, and as a result there is still, even now, considerable misunderstanding in regard to the occurrence and extent of the Pasteur effect in tumors. There is a fallacy extant that malignant tumors are generally low in Pasteur effect, and not uncommonly completely deficient. Part of this has come about from the fact that the Pasteur effect can be, or has been, expressed quantitatively in several different ways, depending upon the particular interest of the analyst, as will now be indicated.

Columns IV to X, Tables VII and VIII, illustrate several direct and derived expressions of the Pasteur effect, the most commonly employed being the absolute inhibition of fermentation (Col. IV), the percentage inhibition of fermentation (Col. V), and the (Meyerhof) oxidation quotient (Col. VI). The simplest, most fundamental, and least committal is certainly the absolute inhibition, expressed in terms of experimentally observed decrease in cleavage product[6] formation due to aerobiosis. The percentage inhibition and oxidation quotients and other measures attempt to give an expression relative to some standard or concept; thus, the first named with reference to anaerobic glycolysis itself, the oxidation quotient

[6] It is important to remember with Warburg (139a, p. 139), "When we speak of a cleavage-metabolism, we have in mind only the final products which actually appear, and take no account of decomposition *phases*, which occur in an inner cycle and disappear again."

with reference to the idea that respiration *per se* is the cause of decreased fermentation, etc. There is good justification for employing any given quantitative measure from occasion to occasion, but the basic nature of the absolute expression of Column IV must never be lost sight of. It will be seen that with most functions there is a gradual *increase* in numerical value as one proceeds through the tissues arranged in the order: stable (non-glandular and glandular), growing, benign, and malignant, although it may not be necessary to reach the end of the order before a given maximum is attained. The most striking regularities of increase are obtained in Columns II, III, VII-X. The percentage of aerobic fermentation in Col. V decreases, but this function is more one of mathematical convenience than of any fundamental significance. The two main functions, absolute fermentation inhibition (Col. IV) and the Meyerhof oxidation quotient (Col. VI), show a moderate increase through the order, mainly accomplished even before growing tissues are reached. From the behavior of these last two most important functions one can definitely say that the Pasteur effect, generally considered, is as marked or more marked in malignant tumors than in the other tissue types. How then arose the above-referred to, common, even if not prevailing, view to the contrary? Reference is made here to more than the superficial decreased percentage

TABLE IX.   The Respiratory Quotient and Anaerobic Glycolysis of Normal Adult and Growing (or Nerve) Tissues and Various Tumors. [a]

Tissue	Animal	R. Q.		$QN_2{}_{CO_2}$
		(in PO_4)	(in HCO_3)	(in HCO_3)
Adult				
Liver	rat	0.79	0.71 [0.72]	(3)
Intestinal mucosa	"	0.85		(4)
Kidney	"	0.86	0.83	(3)
Submaxillary	"	0.87		7
Spleen	"	0.89		(8)
Testis	"	0.94	0.90 [0.93]	(8)
Growing (or Nerve)				
Embryo, 10 mg.	"	1.04		(8)
Embryo	chick	1.00	0.99	(18)
Brain cortex	rat	1.00	0.98	(19)
Chorion (embryo 30 mg.)	"	1.02	1.00	(32)
Retina	"	1.00	1.01	(88)
Tumor				
Jensen sarcoma	"	0.83	0.78 [0.79]	(34)
Breast carcinoma SS 371	man	0.84		7.1[b]
Bladder papilloma SS 365	"	0.86		3.4[b]
Sarcoma 37S	mouse	0.86		27
Spontaneous No. II	"	0.87		16
Tar carcinoma	"	0.87		22
Crocker sarcoma	"	0.89		22
Spindle-cell tar tumor 173	"	0.91		21
Spontaneous No. I	"	0.91		20
Rous sarcoma	chicken	0.92		(30)
Slow-growing sarcoma	rat	0.94	0.77 [0.77]	18
(Pathologic)				
Tubercular lymphatic	man	0.91		14

[a] Ringer- (0.2 p.c.) glucose, pH 7.4, 38° C. Parenthetical values from Warburg, etc. (*cf.* Table VII); all other values from Dickens and Simer (38), with bracketted values of R.Q. obtained by them in serum.

[b] Low, due to admixture with normal tissue.

inhibition, noted in Column V. Paradoxically, Warburg is mainly responsible.

Initially Warburg had stressed the properties of anaerobic and then aerobic glycolysis as the most suitable criteria for characterizing tumor metabolism. But toward the end of his investigations he found that quantitatively the aerobic glycolysis depended too much upon experimental conditions, and that at times (often in embryos) the aerobic glycolysis might be small or negligible *in vivo* even though considerable *in vitro* in Ringer's solution or in serum. The chief reason for this variability was the great sensitivity of the Pasteur reaction compared with respiration or fermentation to general inhibitors. Warburg then made a classification in which a full Meyerhof oxidation quotient of 6 was assumed, and based upon a relation between two measured quantities, $Q^{N_2}{}_{CO_2} - 2Q_{O_2} = U$, termed the fermentation excess (*cf.* Col. IX, Tables VII and VIII). With this experimental, though in some measure arbitrary relation, he found that malignant and non-malignant tumors were the only tissues which gave *positive* U values (*cf.* Col. IX and X), with the rather unimportant exception of retina, which could be otherwise distinguished metabolically by, among others, its very high respiration rate. Nevertheless, with the arrival of the data of Murphy and Hawkins, and of Crabtree (Table VIII), it became evident that certain mouse tumors had negative U values, whereupon Warburg abandoned his U classification, an action which I believe can properly be labelled scientific bending over backwards; way over backwards, because instead of being able to straighten up his next move was to fall down, in short, one (in fact, two as we shall see) of his few mistakes (mistakes of importance).

Up to this point Warburg had always maintained that the respiration of tumors was defective, but sure enough only in the sense of being too low in comparison to the anaerobic fermentation to eliminate aerobic fermentation even after allowing a maximum Meyerhof oxidation quotient of 6 to be operating; he had considered the effect of respiration in reducing fermentation to be normal, namely a Meyerhof oxidation quotient of the common value of 3 to 6. He had worked mainly with human and rat tumors and invariably found positive U values, but now the mouse tumors were found to have a comparatively high respiration, and this was mainly responsible for the negative U values found among certain of the mouse tumors, since the aerobic and anaerobic glycolyses were scarcely different. So, citing the work of Murphy and Hawkins and of Crabtree in Table VIII, but giving only Columns I, II, and III thereof (though by individual experiments, not by averages), Warburg said (139a, p. ix-x, June

1930), as practically his next to last paragraph on cancer metabolism,

"Aerobic glycolysis results if the respiration of growing cells is injured, whether by diminishing its extent or by interfering with the relationship which holds between respiration and fermentation (glycolysis). In Flexner-Jobling rat carcinoma and in the human spontaneous tumours the respiration is abnormally small. In the mouse tumours of Murphy and Crabtree the respiration is certainly large, *but its property of influencing fermentation has been destroyed.*" (Writer's italics).

Shortly before he had said likewise (139a, p. *327*),

"Whether the respiration of the tumor cell is large or small, aerobic glycolysis is present in every case. The respiration is always disturbed, inasmuch as it is incapable of causing the disappearance of the fermentation (*i.e.* glycolysis). Thus the two kinds of disturbances of respiration which can be artificially produced in normal cells—limiting the *extent* of respiration, or hindering the *effect* of respiration—occur in nature, in tumours."

The italicized portion of the first quotation, and the portions of either quotation in accordance therewith, are, I believe, quite incorrect (no less) and without experimental justification or support. Warburg did not give the oxidation quotient, which is a deciding function in this matter, and as the further calculations in Table VIII (Col. VI) show, the Meyerhof oxidation quotients are by no means zero (or quite low), as would be implied by Warburg's view, but they are essentially normal, ranging around 3 (*cf.* also the Dickens-Simer data, etc. of the same Table). The effect of respiration in diminishing glycolysis is obviously quite normal throughout all of the tumor data of Tables VII and VIII (and X) and, so far as I am aware, throughout all significant tumor data available otherwise (though this is not to say that an occasional or isolated instance is not to be met with, where paucity of data gainsays certainty).[7]

How Warburg came to make this error of judgment is a matter of more than passing interest in view of future developments. It is to be inferred that, upon being presented by the rest of the world with the deviating mouse tumor

[7] Warburg's error of judgment here is all the more remarkable in view of the quotation from Clerk Maxwell from Theory of Heat that he gives only two paragraphs after the first of the foregoing quotations, in the Foreword to his book (139a), "The most important step in the progress of every science is the measurement of quantities. Those whose curiosity is satisfied with observing what happens have occasionally done service by directing the attention of others to phenomena they have seen; but it is to those who endeavour to find out how much there is of anything that we owe all the great advances in our knowledge." If only Warburg had stuck more closely to his quotation! As a matter of fact, Maxwell's statement, which reads well, does not bear too close scrutiny (*cf.* David Lindsay Watson, Scientists Are Human, London, Watts & Co., 1938).

respiration data, and clearly sensing that the respiration deficiency which he had always talked about could not here be one of *extent*, he chose the only other main alternative which he had earlier entertained as a possibility but not actuality, namely *effect* of respiration; withal absentmindedly (if such is conceivable) neglecting to check his surmise *via* oxidation quotients. Very possibly he would never have fallen back on the *effect* explanation if during his previous experimental work he had not made a second error, referred to already, this time one of omission, not of commission. In all his experimental work he had failed to observe that the respiration defect in tumors also involved a defective carbohydrate oxidation as expressed by low respiratory quotients (indeed in most of his experimental measurements it was rather necessary to assume that the R.Q. was unity). This important generality was established, shortly after Warburg relinquished his experimental grip on the field, by Dickens and co-workers in a long series of papers, some of the most pertinent data of which is given in Table IX.

Table IX shows that as a generality the R.Q. of tumors ranges from about 0.75 to 0.9, as with most adult tissues, but unlike growing or nerve tissue with which tumor tissue might otherwise easily be confused on a metabolic basis. Whereas in normal, adult, growing, or nerve tissue the R.Q. and anaerobic fermentation increase together, in tumors the low R.Q. and high anaerobic fermentation accompany each other. Metabolically, then, from the work of the Warburg-Dickens schools, *tumors are growing glycolysing tissues with low respiratory quotients*. I know of no interesting exception to this specific, very simple, characterization of tumor, as distinguished from normal healthy, tissue metabolism, though the pathological tuberculous lymph gland instance in Table VIII is to be noted. Embryo and especially retina, which are often claimed to have tumor metabolism, are easily separated upon the basis of their high respiratory rate, or respiratory quotient values of approximately unity corresponding to carbohydrate oxidation. As already stated, the significance of Dickens' finding has been distinctly overshadowed by not only Warburg's positive findings but also his error of commission in regard to the *effect* of respiration. A good deal of the responsibility for this goes, indeed, to Dickens, who readily accepted and perpetrated the *effect* explanation of Warburg, unjudiciously at the expense of his own profound finding, with the result that the importance of the latter is even now by no means generally appreciated. After all, what could the general biochemist do, with Warburg and Dickens both holding out so strongly for the defective respiration explanations of *extent* and

TABLE X. A Comparison of the Development of Dye-Induced Rat Hepatoma and Metabolic Characteristics of Tumors (Nakatani, Nakano, and Ohara, 1938 (104))

Tissue	Days	I Q_{O_2} Respiration	II $Q^{O_2}_{CO_2}$ Aerobic glycolysis	III $Q^{N_2}_{CO_2}$ Anaerobic glycolysis	IV $Q^{N_2}_{CO_2} - Q^{O_2}_{CO_2}$ Glycolysis inhibition (absolute Pasteur effect)	V $100\left(\frac{Q^{N_2}_{CO_2} - Q^{O_2}_{CO_2}}{Q^{N_2}_{CO_2}}\right)$ Percent inhibition of glycolysis by air (p.c. Pasteur effect)	VI $\frac{Q^{N_2}_{CO_2} - Q^{O_2}_{CO_2}}{Q_{O_2}}$ Meyerhof oxidation quotient	VII $\frac{Q^{N_2}_{CO_2}/3}{Q_{O_2}}$ Anaerobic glycolysis Equivalent respiration	VIII $\frac{Q^{O_2}_{CO_2}/3}{Q_{O_2}}$ Aerobic glycolysis Equivalent respiration	IX $Q^{N_2}_{CO_2} - 2Q_{O_2}$ Fermentation-excess, U	X $Q^{N_2}_{CO_2} - 2Q_{O_2}$ Aerobic Glycolysis Fermentation Excess
1. Normal liver	0	12.0	0.5	3.5	3.0	86	0.7	0.9	0.1	−20.5	−5.8
2. o-Aminoazotoluol liver	240	10.1	2.0	9.2	7.2	78	2.1	2.8	0.6	−11.0	−1.2
3. Butter Yellow[a] liver	50	11.6	0.1	10.9	10.8	99	2.8	3.1	0.03	−12.3	−1.1
4. B. Y. liver (non-carc. part)	100	10.7	1.1	9.3	8.2	88	2.3	2.7	0.3	−12.1	−1.3
5. B. Y. carcinoma of liver	100	11.8	8.3	18.8	10.5	56	2.7	5.2	0.2	−4.8	−0.3

a dimethylaminoazobenzene

TABLE XI

Demonstration of the Pasteur-Warburg Reaction by Specific Inhibition
(Warburg, 139, p. 238).

Function	Q_{O_2} [a]		$Q^{O_2}_{CO_2}$ [b]		$Q^{N_2}_{CO_2}$ [c]	
Cyanide-ethyl ester M/1000	—	+	—	+	—	+
Column	I	II	III	IV	V	VI
Jensen rat sarcoma	13.2	13.8	14.6—19.0[e]	28.4—31.6[f]	26.2—27.6	26.2
Liver	10.4	11.2	0	0.6		
Kidney	23.7	25.7	0	6.0		
Testis	11.4	11.2	5.6	8.4	(8)[d]	
Embryo	13.6	13.6	5.3	12.2	(13—23)[d]	

[a] Q_{O_2} (M/100 HCN) = 0—1, all tissues.

[b] $Q^{O_2}_{CO_2}$ and $Q^{N_2}_{CO_2}$ unaffected by M/1000 $(CH_3)_2CHOH_2CN$ (valeronitrile) in sarcoma.

[c] $Q^{N_2}_{CO_2}$ (M/1000 HCN) = 27 for sarcoma.

[e] Meyerhof oxidation quotient, 3.

[f] Meyerhof oxidation quotient, 0.

effect, and Dickens since the early thirties not following up his over-all observation with concentrated studies on its further inner meaning? I say, in 1939, that the defective respiration of tumors is one of *extent*[8] and *low R.Q.,* not of *effect.* Dean Burk has spoken.

[8] As one further remark in connection with the *extent* explanation in connection with the various functions of Tables, VII, VIII, and X (and apart from R.Q. considerations), it seems to me that the expression "anaerobic glycolysis/equivalent respiration" (Col. VII) is one of the best single indicators of tumor metabolism, even if not entirely sufficient. It will be seen that a value of about 6 or greater is indicative of tumor metabolism, the majority of tumors being well above 10, though the mouse tumors again represent border line cases. Even the much disputed case of retina is only 8 to 9. In all of these functions, including Warburg's abandoned U classification, I think it is important not to be looking for perfectly 100 per cent, non-statistical specificity, but for statistical approximations. There is a certain immaturity about looking for the absolute differentiations and then being over-disappointed as subsequent work slowly but surely breaks them down into various exceptions, which exceptions, to be sure, generally have some incidental basis. Thus, observe Warburg when he gave up his aerobic glycolysis basis: "aerobic glycolysis is not specific for tumors. On the contrary, when the respiration is disturbed, aerobic glycolysis occurs in all types of cell. Anyone who is looking for reactions like the Wassermann reaction, must be disappointed by investigations, of which the most important result—after the discovery of tumor metabolism—is the derivation of this metabolism from the metabolic components of the normal cell." (139a, p. 227).

In recent years a much broader base for the Dickens finding has been provided by the Shorr group and many others (*cf.* last section) studying the low R.Q. values in many adult tissues, though no close connection with cancer work has been attempted. Another modern development, growing out of the Warburg findings and combined with the work on chemically induced tumors, has been the observance of the time-course development *in vivo* of tumor metabolism in liver cancers induced by dimethylaminoazobenzene (Butter Yellow dye), as indicated in Table X, in work due to Japanese investigators (*cf.* also Kinosita, (70)). Here the main observations have been unchanged respiration rate, gradual increase of anaerobic glycolysis during Butter Yellow administration throughout the period of about 75 (± 25) days required for the establishment of the malignant tumors, and a sudden development of a large aerobic glycolysis coincident with the actual onset of cancer. The latter observation would be a most striking confirmation of Warburg's finding from a kinetic, developmental point of view, and is too important to be accepted unreservedly without further independent confirmation.

Most of the post-Warburg-Dickens tumor work of the twenties and early thirties has been concerned with experiments on acceleration and inhibition of aerobic glycolysis, and, as can be seen from Table II, tumors command the bulk of interest here. Representative experimentation is

shown in Tables XI to XV. Whereas some of the affecting agents often influence respiration, aerobic fermentation, and anaerobic fermentation differently, there has so far been little specificity in regard to effects on tumors as compared with other tissues, the most striking positive instance being that of potassium ferricyanide on aerobic glycolysis of Balogh tumor, where the Pasteur effect is increased, in contrast to no effect on anaerobic glycolysis, or on aerobic or anaerobic glycolysis in rat, cat, or guinea pig kidney medulla (Mendel and Strelitz, (92)). The interesting inhibitions by glyceric aldehyde (Tables XIV and XV) are probably non-specific in regard to tumor metabolism; the frequent decreases in respiratory quotient caused by this agent should be noted. The findings in Table XV are numerous, but miscellaneous and difficult to correlate and generalize; the influence of the different enantiomorphs, and also dimeric *vs* monomeric forms (Needham, Lehmann), probably enter in a complicated and uncertain manner that needs further working out.

The Search for the Ultimate Loci of the Pasteur Effect (Oxidative Short Circuits or Shunts of Fermentation).

Table XI represents the beginning of the search for the "Pasteur enzyme", the agent of the Pasteur-Warburg reaction. In these experiments Warburg further sharpened the quantitative relations and reality of the reaction by means of a study of reversible specific inhibition and its effect on the Meyerhof quotient. As indicated, M/1000

TABLE XII

Metabolism of Different Tissues Under Low Oxygen Tension. Ringer-bicarbonate, 0.2 p.c. glucose (Laser, 78).

Warburg's differential method = W. Dixon-Keilin method = D.K.

Material	Method	Gas in vol. % in addition to 5% CO_2		Q_{O_2}	$Q_{CO_2}^{O_2}$	R.Q. $\left(\dfrac{CO_2}{O_2}\right)$
		O_2	N_2			
Retina (rat)	W.	95	—	30-32	45·0	0.85
		10	85	29·2	68·1	
		10	85	35·0	70·8	
		5	90	27·85	71·0	
		5	90	29·0	67·1	0.72
Retina (pig)	D.K.	95	—	15	9·3	0.80
		5	90	21	14·9	0.66
		95	—	20·4	14·4	
		5	90	18·5	28·0	
Chorion (rat)	W.	95	—	13·5	1·1	1.05
		5	90	15·4	6·2	
		5	90	16·7	5·9	0.70
Chorion (rat)	D.K.	95	—	25·5	± 0	
		5	90	22·0	10·7	
Allantois (chicken)	W.	95	—	22·3	0	
		5	90	20·5	13·6	
Crocker (mouse) sarcoma	W.	95	—	8·6	17·2	
		95	—	10·8	14·8	
		5	90	8·4	24·9	
		5	90	10·65	29·6	

TABLE XIII

Influence of Potassium Ferricyanide on Aerobic and Anaerobic Glycolysis by Tissues and Mouse Tumor (Mendel and Strelitz, 92).

Tissue	Mol/L.	$Q^{O_2}_{CO_2}$ (Aerobic)	$Q^{N_2}_{CO_2}$ (Anaerobic)
Kidney Medulla			
Rat	0	11.7	26.6
	0.01	12.4	26.2
Cat	0	15.7	
	0.01	16.1	
Guinea Pig	0	14.2	19.7
	0.01	15.3	20.5
Balogh Tumor	0	22.1	28.9
	0.01	2.3	27.5

cyanide ethyl ester inhibits neither respiration Columns I and II) nor anaerobic fermentation (Columns V and VI), but the Pasteur effect is largely inhibited, since the aerobic glycolysis (Columns III and IV) is greatly and reversibly inhibited. In the case of tumor the aerobic fermentation was restored to the full anaerobic value (100 per cent inhibition of the Pasteur effect), and the Meyerhof oxidation quotient reduced from 3 to 0. This is beautifully sharp separation of a coupled reaction from its two components. The certainty of reaction (not tumor!) specificity was further increased by the fact that valeronitrile, a stronger indifferent narcotic for respiration, affected neither aerobic nor anaerobic fermentation. HCN likewise affected respiration a great deal more than aerobic fermentation, contrary to the case of the cyanide ethyl ester. Warburg believed that both HCN and HCN-ester here form, in respiration and in the Pasteur reaction, respectively, different compounds with iron catalysts involved in both cases. Genevois (60) could show for the special case of algae, where respiration is little affected by HCN, that M/1000 HCN inhibited the Pasteur effect, again indicating iron or heavy metal catalysis. Davis (Biochem. Z., *265*, 90, 1933) confirmed this in quite another way by showing that in *B. Delbrückii*, which forms H_2O_2 in a respiration not inhibited by HCN, the Pasteur effect is likewise not affected—no heavy metal catalysis, no HCN inhibition! Krah (73) and Eichholtz (48), using inhibitors claimed to be specific for different heavy metals, also concluded that a ferro-catalysis was involved. The work of Laser (78, 79; *cf.* Table XII) on the increase of

aerobic glycolysis (inhibition of Pasteur effect) caused by either lowering of oxygen tension or presence of carbon monoxide, and counter-inhibition of the latter by light, is strongly suggestive of heavy metal catalysis of the Pasteur effect. (As in Warburg's cyanide ester inhibition, there was practically no effect on respiration or anaerobic fermentation). Whether the O_2 and CO exert their effect at the same point remains for the present a quite open question, and the mere fact that Laser's articles on each gas were published next to each other in the same number of a journal should not necessarily influence one's thinking here. For proof of a connection it will obviously be necessary, as in the case of the *Atmungsferment* earlier, to show a kinetic, *quantitative* competitive action between the two gases. It is to be hoped that Stern and Melnick will be able to obtain evidence here, in their search for the existence, nature, and possible isolation of the Pasteur agent concerned at this point. That some sort of heavy metal catalyst is almost certainly involved follows from all of the evidence just reviewed. It is not without interest that Lipmann, in his first article on the Pasteur effect, in which he studied cell-free extracts, suggested that a cytochrome was very possibly the agent being sought.

"On the basis of the fact that the oxidized glycolytic ferment is inactive, the following may be said: The ferment is not autoxidizable, since muscle extract, which contains no *Atmungsferment*, glycolyzes as well in the presence as in the absence of oxygen. If indophenol is substituted in the extract for the lacking *Atmungsferment*, then the glycolysis in oxygen will be inhibited here also. *In the cell the ferment is inactivated in the presence of oxygen through the agency of the autoxidizable Atmungsferment, which inactivation reaction is called the Pasteur reaction.* In the presence of cyanide the cell glycolyzes, since after blocking of the autoxi-

TABLE XIV

Influence of *dl*-Glyceric Aldehyde on Aerobic and Anaerobic Glycolysis by Tissues and Tumor (Mendel and Strelitz, 92)

Tissue	Mol/L.	$Q^{O_2}_{CO_2}$ (Aerobic)	$Q^{N_2}_{CO_2}$ (Anaerobic)
Kidney Medulla			
Rat	0	14.2	
	0.004	2.3	
Cat	0		14.6
	0.002		1.9
Balogh Tumor	0	19.9	32.8
Mouse	0.002		2.8
	0.004	3.7	

dizable system the glycolytic ferment will be reactivated by the reduction systems of the cell. And since it is to be supposed that both of the energy-yielding reaction ferment catalysts are not freely diffusable in the cell, it is scarcely to be considered that the oxidation of the glycolytic occurs by direct contact with the *Atmungsferment*, but much more probably by means of a freely diffusible carrier substance. As such, one of the cytochromes could function." (82, p. 139).

This was written before the work of Laser on the influence of oxygen pressure was done, and if the Pasteur agent here turns out to be a WKS (Warburg-Keilin System) component, or one very similar thereto, it will be interesting as to whether it is indeed autoxidizable, as well as affected by carbon monoxide, and even other inhibitors indicated in Table II.

The evidence in favor of obtaining a Pasteur effect by means of various heavy metal poisons is abundantly clear, and suggestive of an effect operating someplace or in several places in the Warburg-Keilin System. But this is only one special region of possible causation of effect, and there remains a vast territory covered by the fermentation systems, as Table XVI will, I hope, bring out with some force. Just as we saw in Table I that no one Pasteur effect explanation would suffice, so no one locus will suffice, though some explanations or loci may be more fundamental and less incidental than others. In regard to loci, too, one must distinguish between the exterior acting experimental causes, which may be many and various, and the ultimate loci of influence, which will

TABLE XV

The Effect of *dl*-Glyceric Aldehyde on the Metabolism of Glycolysing Tissues (Baker, 5).

Tissue	Conc. (M.)	$Q^{N_2}_{CO_2}$ (Anaer. Gly.)	$Q^{O_2}_{CO_2}$ (Aer. Gly.)	Q_{O_2} (Resp.)	R.Q. (Resp. Quot.)
Sarcoma (Phil. I)	0	35.4	30.3	11.7	0.74
	0.0005	29.0(—18)			
	0.0010	12.7(—36)			
	0.0022	2.2(—94)	18.2(—40)	10.6(— 9)	0.73
	0.01		7.5(—75)	5.8(—50)	0.42(—43)
	0.05		4.6(—85)	4.3(—63)	
Brain	0	10.0	1.0	14.8	0.93
	0.0022	0.5(—95)	1 9	12.9(—13)	0.90
	0.011	0.4(—96)	1.2	5.0(—66)	0.67(—33)
Testis	0	12.4	9.2	13.6	0.91
	0.0022	8.8(—29)	10.7(+17)	7.2(— 9)	0.83
	0.011	1.9(—84)	10.9(+20)	7.2(—46)	0.81(—10)
	0.05		7.7(—15)	3.9(—71)	
Whole Chick Embryo, 5-day	0	22.5	3.4	14.0	0.98
	0.0022	8.6(—62)			
	0.011	1.1(—95)	2.2	13.0(— 7)	1.04
	0.05		4.0	4.0(—71)	0.86(—12)
Liver	0	1.7	0.7	11.6	0.77
	0.011	5.5(+225)	2.7(+280)	12.5(+ 8)	0.70(— 9)
	0.05	8.4(+395)	9.8(+1300)	6.6(—43)	0.53(—31)
Kidney	0	2.7	1.4	27.0	0.81
	0.011	2.5	4.0(+185)	29.0	0.78
	0.05	3.6	8.5(+520)	25.0	0.77

Parenthetical values indicate percentage loss or gain.

be fewer though still probably various, and certainly determining in part by kinetic considerations, as emphasized by Barron.

The general problem appears to develop into a question of where in some such series of reactions as numbers 1 to 14, Table XVI, the fermentation is shunted or short circuited under aerobic conditions. One can think here of various acting agents, O_2 gas itself, the WKS, flavoproteins, or other autoxidizable agents, all functioning in one or different ways under aerobic conditions. One of the most concrete proposals as to the ultimate point of their action is at the pyruvic acid stage where cozymase is concerned, and this may be regarded in its various forms and details as a particularization of the Lipmann generality (reviewed in Table XVII; and cf. Table I) expressed as glycolytic ferment. The schema of Ball, Table XVIII, is an excellent setting forth of the relations in pictorial (even if not kinetic) detail. There is a competition, in short, at pyruvic acid (or acetaldehyde) for oxidation on the one hand, or reduction (to lactic acid or alcohol) on the other. The determining external condition is the presence of oxygen, and thus oxidized states within the WKS and flavoprotein systems; and the determining internal condition is the degree of oxidation-reduction of the cozymase (glycolytic) ferment system. There are many ways of describing the situation portrayed by the Ball schema, and much more might be said in detail. Table XIX gives further expansion in relation to amino acid synthesis, but with the same competition principles of the O_2-WKS-flavoprotein system keeping cozymase oxidized, and with the amino acid metabolism corresponding to the fermentation metabolism in Table XVIII. Table XX is a similar schema involving the amino acid acetylation synthesis of duVigneaud-Irish (46), and Table XXI is likewise a schema placing the emphasis of the shunt locus at the pyruvate stage.

For other shunt loci, it is now possible to climb up Table XVI, so to speak, and as noted in Table I (mainly but not only under Shunt-Loci) there are a number of definite bids and indications already staked out by various investigators. Most skip up at least to Stage 7-8, at which cozymase is involved exactly as at Stage 13-14. The Szent-Györgyi schema (Table XXII) calls for separation at the triose stage, and is more concerned with substrate fate at the point of separation than with enzymes concerned. Thus, most of those at Stage 7-8 involve logic based on the action of the inhibitor iodoacetate, universally admitted to affect triosephosphate dehydrogenase, probably the protein rather than the cozymase part (Rapkine, Gemmill-Hellerman). This inhibitor is indeed remarkable in being able, like few others, to clean-

ly prevent lactic acid or alcohol formation without affecting respiration (due care being taken with regard to concentration). There is a long and very interesting literature involved that cannot be given here, but reference may be made to the various Shorr papers on animal tissues already referred to, and in particular Ref. (135, especially Fig. 3 and 4) or (9, especially Fig. 2 and 3), and to the Lundsgaard (88, 89, 90) and Boysen-Jensen (16) papers on yeast (cf. Table XXIII), and to Krebs (75), Quastel and Wheatley (113), Meyerhof and Boyland (97), Stannard (130), Saslow (120), Himwich and Fazekas (66), and Baker, Fazekas, and Himwich (J. Biol. Chem., 125, 945, 1938). Various of these shunts have been discussed thoroughly elsewhere in the Symposium, and I shall therefore go on to one which has been little considered (in connection with the Pasteur effect) but which I believe deserves great emphasis, namely that shunt at hexosemonophosphate (Stage 3) as proposed by Engelhardt and Barkash (49, 50).

The fate of hexosemonophosphate upon oxidation has been studied mainly by Lipmann (83), Engelhardt and Barkash (49, 50 and later), Warburg and Christian (Biochem. Z., 287, 302, 1936, and 292, 287, 1937), and by Dickens (35). It remained for Engelhardt and Barkash to very concretely propose a Pasteur effect shunt at the hexosemonophosphate stage; Engelhardt writes as follows (49):

"My opinion is that hexosemonphosphate represents the point where the fate of the hexose molecule is determined and the paths of respiration and fermentation diverge. If the monoester undergoes further phosphorylation to the hexosediphosphate then the hexose molecule is brought into the route of fermentation, beginning with the splitting of the carbon chain in C_3 fragments. If, on the contrary, the monophosphate instead of being phosphorylated at the C_1 is oxidized at this very place with formation of phosphohexonic acid, then the molecule is destined to oxidative breakdown:

$$+H_3PO_4 \longrightarrow \text{hexosediphosphate} \longrightarrow \text{(fermentation)}$$
$$-H_2O$$
$$\text{hexosemonophosphate}$$
$$+H_2O$$
$$-2H \longrightarrow \text{phosphogluconic acid} \longrightarrow \text{(oxidation)}$$
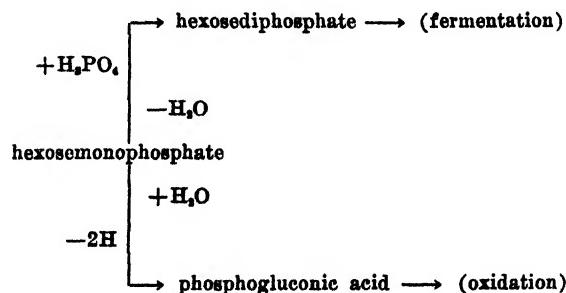
The oxidative breakdown would proceed not through cleavage of the C_6 molecule into two C_3 halves, but, as suggested by Lipmann, through gradual stepwise shortening of the carbon chain until the three-carbon stage is reached.

TABLE XVI. A (1939) Working, Fairly Stabilized Schema of Intermediate Carbohydrate Fermentation in Muscle or Yeast, Etc., With Energetics (Embden-Meyerhof-Parnas-Needham-Cori-Warburg Schools).

4. DIHYDROXYACETONE PHOSPHATE(Meyerhof-Lohmann-Kiessling ester)

$CH_2OPO_3H_2$
CO
CH_2OH
(opt.inact.)

isomerisation
isomerase
(< 5%,20°C)

(reduction,in F or B_2O_3)
α-GLYCEROPHOSPHATE (Lohmann ester II)
$CH_2OPO_3H_2$
$CHOH$
CH_2OH
(dephosphorylation)
(+ H_2O)
GLYCERIN($C_3H_8O_3$)+ H_3PO_4

7. 3-PHOSPHOGLYCERIC ALDEHYDE(Fischer-Baer ester)

$CH_2OPO_3H_2$
$CHOH$
CHO
(d-rot.)

phosphorolysis
enzyme?
(trace %)

(secondary decomp.)
(-H_3PO_4)
METHYL GLYOXAL(CH_3COCHO)
+H_2O
(alkali, 20°C)
(glyoxalase,glutathione as coenzyme)
LACTIC ACID($CH_3CHOHCOOH$)
+ INORGANIC PHOSPHATE

8. 1-2-DIPHOSPHOGLYCERIC ALDEHYDE(Warburg-Christian ester)

$CH_2OPO_3H_2$
$CHOH$
$CHOH$
OPO_3H_2

dehydrogenation
triose diphosphate dehydrogenase(CH_2ICOOH poisoning)
(Co-protein → Co-H_2-protein; + pyruvate or acetaldehyde → Co-protein + lactate or alcohol)

9. 1-3-DIPHOSPHOGLYCERIC ACID(Negelein-Brömel ester)

$CH_2OPO_3H_2$
$CHOH$
CO
OPO_3H_2

dephosphorylation
phosphorylase

10. 3-PHOSPHOGLYCERIC ACID(Nilsson-Lohmann I ester)

$CH_2OPO_3H_2$
$CHOH$
$COOH$
(l-rot.)

shift
phosphoglyceromutase
(no coenzyme?)
(20%,20°C)

11. 2-PHOSPHOGLYCERIC ACID(Kiessling ester)

CH_2OH
$CHOPO_3H_2$
$COOH$
(d-rot.)

dehydration
enolase(no coenzyme?)
(fluoride inhibition)
(30%,temp. insensitive)

12. PHOSPHOPYRUVIC ACID(Lohmann-Kiessling ester)

CH_2
$COPO_3H_2$
$COOH$
(d-rot.)

dephosphorylation
dephosphorylase

13. PYRUVIC ACID

$CH_3COCOOH$

desarboxylation
carboxylase

14. ACETALDEHYDE

CH_3CHO + CO_2

LACTIC ACID($CH_3CHOHCOOH$)
(via Co≈Co-H_2, B protein; flavoprotein, WB, b_3)
ETHYL ALCOHOL(C_2H_5OH)

Hydrolysis
(in absence of O_2, glycolysis; e.g., with + CH_2ICOOH)
INOSINIC ACID + NH_3

ENERGETICS
(on a 3-carbon compound basis)

Reaction(aqueous)	ΔH(calories)
$(Glycogen)_n$ + $\frac{1}{2}H_2O$ = $\frac{1}{2}$glucose	-2,800
$\frac{1}{2}$glucose = lactic acid	-15,400
$(Glycogen)_n$ = lactic acid	-16,200
(lactic acid + diphos. = lactate + monophos.	-1,700
lactic acid + proteinate = lactate + prot.	-12,600
$(Glycogen)_n$ + 5/7diphos. + 4/7proteinate	-24,200
(as in muscle) = lactate etc.	
$(Glycogen)_n$ + H_3PO_4 = hexosemonophosphate	ca. 0
Hexosemonophos.+H_3PO_4 = hexosediphos. ± H_2O	ca. 0
$(Glycogen)_n$ + H_3PO_4 = hexosediphos. + H_2O	ca. 0
$\frac{1}{2}$Hexosediphos. = dihydroxyacetone phos.	+7,000
Dihydroxyacetone phos.=phosphoglyceric aldehyde	0
$\frac{1}{2}$Dihydroxyacetone phos.+$\frac{1}{2}$glyc.ald.=$\frac{1}{2}$fruct-1-phos.	-7,500
(Dihydroxyacetone = $\frac{1}{2}$hexose (non-enzmic)	-7,500
$\frac{1}{2}$phosphoglyceric aldehyde + $\frac{1}{2}$pyruvic acid	
= $\frac{1}{2}$lactic acid + $\frac{1}{2}$phosphoglyceric acid	-7,000
3-phosphoglyceric acid = 2-phosphoglyceric acid	+5,000
2-phosphoglyceric acid = 2-phosphopyruvic acid + H_2O	0
3-phospho...= 2-phosphopyruvic acid+ H_2O	+5,000
Phosphopyruvic acid + H_2O = pyruvic acid + phos.	-8,600
Phos. + adenylic acid = adenosinetriphosphoric acid	+12,000
ATP + creatine = adenylic acid + phosphocreatine	+12,000
Phosphopyruvic acid + creatine + H_2O = pyruvic acid	0
+phosphocreatine (Parnas reaction)	
Phosphocreatine + H_2O = creatine + phosphate	+5,400
	-12,000

(ΔH is negative when heat is yielded)

TABLE XVII

Qualitative Summary of Three Major Classical Theories of the (Reversible) Pasteur Effect (Burk, 20)

Pasteur-Pfeffer-Pflüger (1900) (Unitary theory)	1. carbohydrate → cleavage product 2. cleavage product $+O_2$ → $CO_2 + H_2O$ *fermentation intermediates oxidized*
Meyerhof (1920-) (Meyerhof cycle theory)	1. carbohydrate → cleavage product 2. cleavage product $+O_2$ → carbohydrate $+ CO_2 + H_2O$ *fermentation intermediates mainly resynthesized*
Lipmann (1933-) (O/R theory)	1. carbohydrate → cleavage product (O_2 inhibits *via* O/R potential of enzymes) 2. carbohydrate (or cleavage product) $+ O_2$ → $CO_2 + H_2O$ *fermentation intermediates mainly not formed*

TABLE XVIII. Carbohydrate Fermentation-Oxidation Schema of Ball (7).
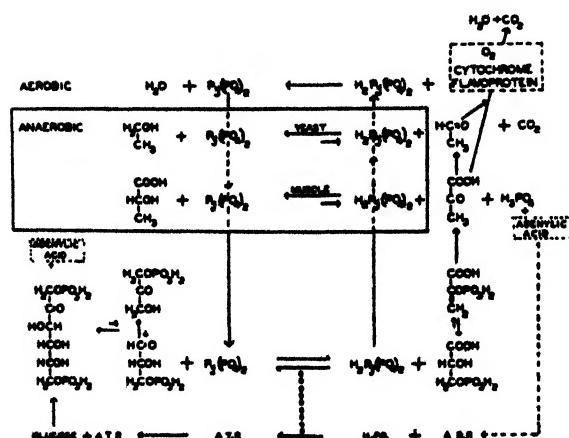
TABLE XIX. Ball's Schema of Amino Acid Oxidation (7).
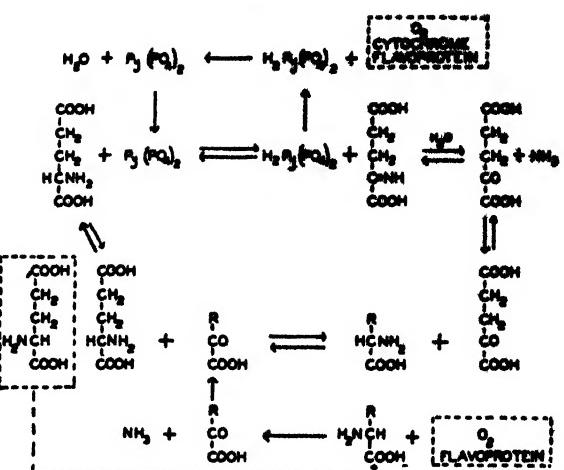
TABLE XX. duVigneaud-Irish Schema of Amino Acid Oxidation and (Acetyl-) Synthesis (46).
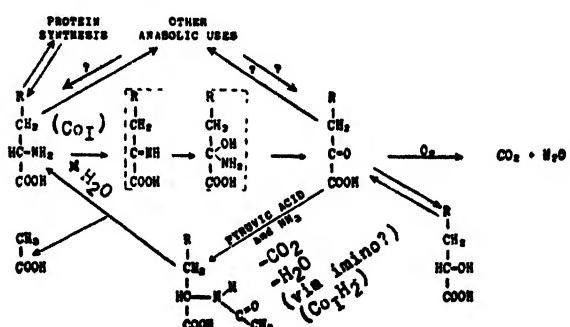
TABLE XXI. Potter-Elvehjem Schema of Catabolism and Anabolism of Carbohydrates (112).
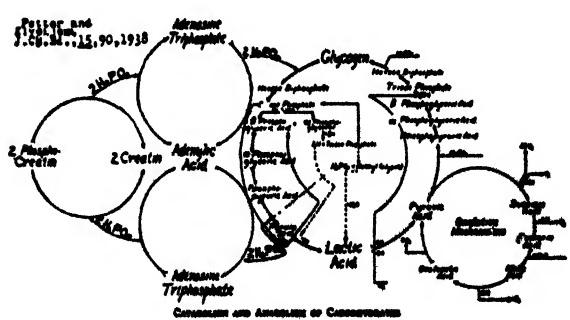
TABLE XXII. Szent-Györgyi Triose Shunt (134).

```
                        CH₃          CH₃
                        |            |
                        CO    →     HCOH
                        |            |
                        COOH         COOH
                        Pyruv.       Lact.
                        ac.          ac.
     H₂COH
     |
     HCOH
     |            H₂COH              CH₃
     HCOH    ⇌   2 HCOH    ─────→    |
     |            |                  CO
     HCOH         HCO                |
     |                               COOH
     HCOH         Triose             Pyruv.
     |                               ac.
     HCO
     Hexose              2H
                      Oxidation ─→  COOH         COOH
                                    |            |
                                    HCH          HCH
                                    |            |      2H
                                    CO    ⇌     HCOH ─────→ Fum.—Succ.—WKS—O₂
                                    |            |
                                    COOH         COOH
                                    oxaloac.     malic
                                    ac.          ac.
```
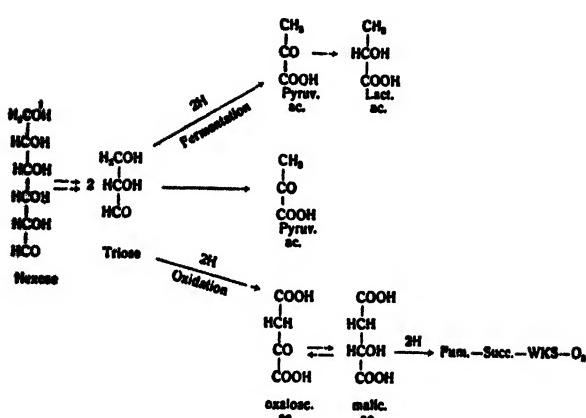
If this view turns out to be correct, then the point of application of the Pasteur reaction is to be sought at the level of hexosemonophosphate. For instance, it could be supposed that a shift of the Lohmann-equilibrium between aldose- and ketose-monophosphate in the direction of more complete formation of the aldose isomer would favour the oxidation and make impossible the phosphorylation to diphosphate, and fermentation would be inhibited. This stands in accord with the explanation given by Michaelis and Smythe of the Lipmann results; I could completely confirm the findings of Michaelis that it is the non-formation of diphosphate which is responsible for the suppression of fermentation in the presence of redox dyes."

The original paper of Engelhardt and Barkash is in Russian (50), but there is an excellent English summary to which reference for supporting details may be strongly recommended. The main points of the evidence include the inability of phosphogluconic acid to undergo non-oxidative (anaerobic) transformations, the small sensitivity of the CO_2 formation towards glycolytic poisons (fluoride and iodoacetate), and the absence of any indications of a splitting of the C_6 chain previous to CO_2 formation (O_2 needed for this). The oxidation of phosphogluconic acid was achieved under anaerobic conditions in the presence of suitable hydrogen acceptors, e.g., methylene blue, oxidized glutathione, dehydroascorbic acid; or at the expense of the juice's own acceptors, if these are oxidized with iodine: thus a C_6 molecule may be burnt to CO_2 by an inorganic oxidizing agent. Acetaldehyde can also act as acceptor in the system studied (yeast maceration juice), evidently being reduced to alcohol; this is formally the same as in fermentation proper: anaerobic formation of CO_2 and alcohol; but virtually the reaction represents a form of linkage between the oxidative and fermentative paths of the breakdown of the sugar molecule. Adenosinetriphosphate is definitely involved in several ways, and non-phosphorylated gluconic acid can be utilized in its presence.

It seems to me that the work of Engelhardt and Barkash (Dickens, Lipmann, Warburg and Christian) has opened up one of the most promising future fields as to the nature of carbohydrate oxidation, especially now that it is definitely established that respiration can proceed unimpeded in appropriate concentrations of iodoacetate and other reagents that practically completely inhibit fermentation beyond a triosephosphate stage. Adler, Euler and Günther (1), moreover, have established the important point that iodoacetate has no effect on hexosemonophosphate apodehydrase. A great deal remains to be determined concerning the further details of the oxidation reaction at Stage 3, Table XVI, especially just at present the nature of the 3-carbon residue.

TABLE XXIII. Influence of Iodoacetic Acid on Metabolism of Yeast (Lundsgaard, 1932) Substrate: Alcohol

	CO_2	O_2	R.Q.	Alcohol (mg. p.c.)				
	mm.³	mm.³		to CO_2	to Acid	Disapp. (calc.)	Disapp. (det.)	To Carbohydrate, (etc.), apparent.
CH₂ICOOH	97	309	0.31	9.9	25.1	34.8	34	—0.8
No CH₂ICOOH	156	443	0.36	16.0	20.6	36.6	52.0	14.0

Oxidation Quotients:	CH₂ICOOH	no CH₂ICOOH
$\dfrac{\text{alcohol disappeared}}{\text{alcohol oxidized}}$	3.4	3.3
$\dfrac{\text{alcohol synthesized (calc.)}}{\text{alcohol oxidized}}$	0	0.9

R.Q. rises to unity (1/2 hr. in no CH₂ICOOH) (3 hrs. in CH₂ICOOH), O_2 and CO_2 falling.

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DISCUSSION

Dr. Lipmann: Burk has already pointed out that it is very important to bear in mind that the Meyerhof quotient is an *experimental* quantity. Now, at least in the cases where the Laser experiment holds, that is, where the inhibition of glycolysis by increasing O_2 pressure occurs at a constant rate of respiration, a simpler kinetic picture is involved and the Meyerhof quotient loses greatly in interest and significance, I believe. I have always been somewhat bothered by the Meyerhof quotient and have thought that as long as a relation between the rate of respiration and the disappearance of fermentation exists, a complicated steady-state must exist between the catalysts acting aerobically and anaerobically, respectively. But if with decreasing oxygen pressure the active respiration can stay at the same level and fermentation can increase, as Laser maintains, then we can really talk confidently about the inhibitory theory of the Pasteur effect, and need

no longer necessarily take the rate of respiration into account.

As to the scheme of Engelhardt, I think it is very interesting, and I started to work on gluconic acid metabolism after Warburg's work on glucose-6-phosphate had appeared. I found that decomposition of phosphogluconic acid occurred only in oxygen. I did the experiments because I thought that here might be a new explanation of the Pasteur effect, since it was possible for another path of oxidation of glucose to occur, starting in this case with hexosemonophosphate, and breaking down the molecule from one end. I did not go far with this work because two weeks after my note came out the very much better work of Warburg appeared showing that hexosemonophosphate can be broken down (from one end) to at least the three-carbon stage. As time goes on I think this alternative method of oxidation, as Burk also seems to think, will give a major explanation of the Pasteur effect, at least in animal tissues. In this connection, Shorr's experiments with Iodoacetate are very important. As he points out, it is otherwise difficult to see how respiration can go on in the absence of fermentation if no other pathway of oxidation is present.

Dr. Gemmill: Since it is necessary to reduce the coenzyme in order that the triosephosphate will go to phosphoglycerate, how will pyruvate be formed if the cozymase is not in the oxidized state?

Dr. Burk: I think this is a question of the relative effects of any given degree of oxidation-reduction of the coenzyme on the formation of pyruvate from glucose and on the continuance of pyruvate to alcohol or lactate. We do not have to assume the coenzyme to be 100 per cent oxidized but only partially so, and the degree of oxidation might quantitatively affect differently the production of pyruvate from glucose as compared with the production of alcohol or lactate from pyruvate. One inserts the idea of variable degrees (and effects) of oxidation-reduction in order to understand the kinetics more readily, and also the existence of the Meyerhof oxidation quotient being greater than unity.

Dr. Gemmill: Would it not be better to put the coenzyme short-circuit at the triosephosphate stage instead of at the pyruvate stage?

Dr. Burk: Yes and no. As I said, there are many points, fine and otherwise, and alternative dispositions, that one might discuss about Ball's scheme or representation; the only one I was giving was Ball's main idea of the Pasteur effect mechanism.

Dr. Gemmill: Are you sure the glycolysis Laser reported was really aerobic glycolysis?

Dr. Burk: No doubts arise in my mind. I think we may regard anything coming out of

Keilin's institute, where Laser worked, as being properly carried out and described.

Dr. Gemmill and Dr. Hellerman: Burk has included us as of the category of experts in the field of the Pasteur effects, an honor which we must respectfully disclaim. We wish, therefore, to point out that we are not, and have never been, committed to any "theory" of the so-called "Pasteur-Meyerhof reaction". Our published work on glycolysis deals with certain observations regarding reversible inhibition, and we have speculated very briefly concerning the factors controlling the glycolytic mechanism in frog muscle. In his paper in this Symposium, Gemmill has reviewed some of the possibilities for short-circuiting the glycolytic mechanism under aerobic conditions.

From a thermodynamic standpoint, the introduction of oxygen (in the presence of the catalytic mechanisms permitting interplay with this otherwise sluggish molecule) into a biological environment, the immediately preceding state of which was essentially anaerobic, would itself constitute so drastic a change as to direct the utilization and disposal of various metabolites (*e.g.*, pyruvate, triosephosphates, and even certain phospho-hexoses) into new channels. Such changes might include the partial "reversal" of glycolysis or, to a greater or lesser extent, its inhibition. The inhibition, in this sense, is merely incidental to glycolytic control. It follows that pat schemas of glycolysis and anaerobic cellular oxidation can easily be made to prove too much, especially when they are transferred from anaerobic to aerobic conditions.

Dr. Burk: Right! These remarks are especially apt and succinct: like the schemas spoken of, pat. They lean in their generality heavily towards the Pasteur-Barron view, or even beyond to the Pasteur (Pasteur-Burk) view. I would point out that the expression "short-circuiting the glycolytic mechanism under aerobic conditions" is an accurate 1939 paraphrase of the Pasteur view "O_2 inhibition of fermentation" and that merely to talk a good deal about this subject is sufficient to quality as a Pasteur effect expert in view of the present prevailing standards. To pass as an expert here is not necessarily the honor that to all appearances Gemmill and Hellerman in good faith affirm. Concerning a theory of the Pasteur-Meyerhof reaction, Gemmill and Hellerman have assuredly never explicitly committed themselves ere this Symposium—the *three* of us are cagey musketeers together in this respect—but at this Symposium they have strongly implicated triosephosphate dehydrogenase inhibition (*cf.* Table I) as one possible shunt for aerobic fermentation. Implication is, of course, by no means explicit commitment.

Dr. Goddard: With reference to Burk's com-

ments on the results of Laser, great caution must be exercised in applying such results to other organisms. This can be illustrated by results obtained by Marsh and myself (Am. J. Bot., in press) on the Pasteur effect in root tissue of carrot. Our experiments show that in this tissue aerobic fermentation can only be obtained when the respiratory inhibition is large (greater than 45 per cent). It is immaterial whether a particular level of respiration has been obtained by lowering the oxygen pressure or by poisoning the respiration with carbon monoxide, cyanide, or azide. These results are consistent with the view that the mechanism of oxygen inhibition of fermentation is by means of cytochrome oxidase.

In carrot root tissue the Meyerhof quotients are high, equal to those obtained with yeast or muscle. I might add that I am not convinced that most higher plants will show low Meyerhof quotients; there is a lack of experimental data on this subject.

Burk has discussed the various locations where the common pathway may branch to give separate pathways of respiration and fermentation. Shorr's paper has clearly shown that in mammalian muscle glycolysis may be separated from respiration by iodoacetate poisoning. This would indicate that the separation must or may occur early, either at the glucose or hexosephosphate stage. We do not know how generally this will be found. Turner (New Phytologist *37*, 289, 1938) found that in carrot tissue both respiration and fermentation were poisoned by low concentrations of iodoacetate, and that it was impossible to poison fermentation without poisoning respiration. Marsh and Goddard (unpublished) have confirmed Turner's observations with iodoacetate and have obtained similar results with NaF. These results indicate that in carrot the common pathway of respiration and fermentation must branch at a fairly low level, perhaps at the triosephosphate or pyruvate stage.

Dr. Burk: I quite agree with what you say. I think the situation in plants can commonly be described as an alcoholic fermentation with ordinary Meyerhof quotients, with often little, if any, actual fermentation in air, except that caused by extreme oxygen lack (anaerobiosis) where the thickness of the plant material prevents adequate O_2 diffusion.

Dr. Stern: May I summarize the evidence for the existence of the Pasteur agent, which, I think, is no contradiction to the term "Pasteur effect"? The "Pasteur effect" is a catalysis or, rather, an anti-catalysis of fermentation, and hence one would expect that there might exist a catalytic agent.

According to Kempner, oxygen reacts directly with one component of the fermentation system. His further explanation for the effect of carbon monoxide in inhibiting the Pasteur effect was that carbon monoxide would displace oxygen from the component of the fermentation system. That means that carbon monoxide would actually activate an enzyme: oxygen and carbon monoxide act on one common intermediate factor betwen oxygen and the fermentation system, which factor I call the Pasteur agent. Under normal conditions of oxygen tension, oxygen would keep this agent in the oxidized state, assuming that it is a reversible oxidation-reduction system. This agent in turn would, in agreement with Lipmann's theory, keep an enzyme of the fermentation system in an oxidized or inactive state. In this manner we can understand why carbon monoxide should have the effect which Laser described. It would combine with the Pasteur agent and would displace the oxygen. Therefore, there would no longer be the check on fermentation.

Now, we can go one step further and form a concept about the chemical constitution of this agent. Burk discussed the effect of ethyl carbylamine on the metabolism of tumor tissue. The anaerobic fermentation was not altered, neither was respiration. All that happened was that the aerobic glycolysis rose to the level of anaerobic glycolysis. Toda, in Warburg's laboratory, examined the effect of ethyl carbylamine on a number of catalytic actions and he came to the conclusion that, in contrast to cyanide, it did not combine with ferric iron; he further showed that ethyl carbylamine did not inhibit catalase. That was in 1926, and it was not known at that time that catalase is a compound existing in the ferric state. Warburg showed later that hemoglobin can combine with methyl carbylamine and that this compound can add one molecule of carbon monoxide, and the resulting complex shows a high light sensitivity. From these various experiments we might infer that ethyl carbylamine combines preferably with ferrous iron.

Now the experiments of Laser indicate that the Pasteur agent can also combine with carbon monoxide, and that this complex is reversibly dissociated by light of ordinary intensity. This again strongly suggests the occurrence of iron in the Pasteur agent. The increase in aerobic glycolysis in the presence of HCN is probably due to complex formation with the ferric form of the Pasteur agent rather than to an indirect effect *via* the inhibition of respiration. In 1929 Warburg published a paper on the spectrum of the respiratory ferment in the retina, in which he endeavored to show that the respiratory ferment in this tissue is identical with the respiratory ferment in yeast. The absorption spectrum of the respiratory ferment-CO complex in yeast and in acetic acid bacteria has an α-band at 592 mμ, a β-band at 540 mμ, and a γ-band at 430 mμ. Warburg was

primarily interested in showing that the ratio of the extinction coefficients at 436 and 405 mμ was the same for retina as it was for yeast. The ratio B_{436}/B_{546}, on the other hand, was found to be 8 for retina and 14 for yeast. In spite of this discrepancy, Warburg's conclusion was that the respiratory ferment in yeast is essentially identical with the respiratory ferment in retina.

The experiments of Laser lead us to reinterpret Warburg's experiments in an entirely different light. Warburg was very possibly measuring, in effect, the absorption spectrum of the Pasteur agent in retina, for the following reason. He was so firmly intrenched in the idea that there is a fixed relation between respiration and fermentation that it is immaterial which is measured in order to get an idea of the operation of the Pasteur effect in any cell. Laser has shown that one can decrease oxygen pressure to 5 per cent without affecting respiration, but the Pasteur action will be inhibited in a manner which is reversibly affected by light.

Now, Warburg did not measure the respiration of retina, but he measured fermentation for methodical reasons, on the postulate that any decrease in fermentation must be due to an increase in respiration, and any increase in fermentation must be due to an inhibition of respiration. Making this assumption he proceeded to irradiate his cells after poisoning them with carbon monoxide, and the change of aerobic glycolysis served simply as an indicator of the effect on respiration; in this way he obtained the three bands mentioned above. Since he was not measuring respiration, and since Laser has shown that respiration is not affected under these conditions, Warburg was very possibly studying the absorption spectrum of the Pasteur agent, and his figures indicate that it is different in the green region from that of the respiratory ferment. It is our plan to go back to Warburg's original experiments, add Laser's new evidence, and try with a large number of wavelengths to map out "the spectrum of the Pasteur agent."

Dr. Shorr: Barker and I have attempted to use ethyl carbylamine in analyzing the mechanism of restoration of carbohydrate metabolism in diabetic tissue *in vitro*. We found that this compound does not abolish the Pasteur effect in cardiac muscle. I might also say, with respect to Stern's comment on the formation of hemoglobin complex by ethyl carbylamine, that we were struck by the peculiar color of the heart muscle exposed to this substance. The color of the muscle changed to a lovely pink.

I would like to bring up one point in connection with one of Burk's statements as to our justification for using extra oxygen consumption due to lactate in the calculation of the lactic acid oxidation quotient, as against the total oxygen consumption. I feel that, at least for the present, we are forced to consider the basal respiration as inhibiting the aerobic appearance of the lactic acid which would appear under anaerobic conditions. The extra oxygen consumption in the presence of lactate would then be dealing with the "extra" disappearance of lactate aerobically.

Dr. Lipmann: I would like to raise a question about Dickens' inhibitors. Phenosafranine and other compounds of this type should scarcely act as inhibitors of metal catalysis, but at some other point. I would like to hear what Stern thinks about this.

Dr. Stern: In principle there is, of course, no reason why there should not be a chain of Pasteur catalysts acting on fermentation. Perhaps we are over-simplifying the picture at the present time. Dickens used phenylhydrazine, and we know from Warburg's previous work that this affects hemoglobin in a characteristic fashion. It is very interesting to see that this reagent inhibits the Pasteur reaction, and that makes me think that perhaps the Pasteur agent not only contains ferrous iron, but also represents a compound of the general type of the respiratory ferment or built after the model of hemoglobin.

Dr. Cori: Is one justified in speaking of a Pasteur effect if one adds lactic acid to a system and finds it to disappear partly by oxidation and partly by synthesis to glycogen and to other products?

Dr. Burk: That is possibly a somewhat artificial experiment; but it is done so commonly and is so deeply ingrained in the literature that, while your very conservative approach here might otherwise be applicable, I do not believe it would be justifiable now in the light of established convention and tradition. Too many people have done such experiments and labelled their results instances of the Pasteur phenomenon.

Dr. Cori: What I wish to point out is the following aspect of the situation. In Meyerhof's main early experiment there was a disappearance of lactic acid which accumulated in muscle as a result of stimulation. He showed that part of the lactic acid disappeared by oxidation and part went to glycogen. The development of the general Pasteur effect concept is due to Warburg later, in whose experiments the resynthesis had no reality if one assumes that the lactic acid formed in N_2 was not formed in O_2. I do not think that these two types of experiment are comparable at all.

Dr. Burk: If we go back to Pasteur I think we find adequate grounds for considering both cases true "Pasteur effects". And we really should go back to Pasteur, skipping Meyerhof and

Warburg (or rather, skipping over them) because Pasteur was really concerned, it can be said, with the general inhibiting effect of oxygen on anything that he happened to measure. I should say that the inhibiting effect of oxygen on whatever the lactate went to (or carbohydrate did not go to) would certainly be within the confines of the broad definition of the Pasteur effect advanced in my paper. If in the last part of my paper I had considered oxidative synthesis in its contemporaneous detail, I believe it would have made the great generality of the Pasteur effect seem even more reasonable and acceptable, because there have been many papers in the last two or three years dealing with this aspect of the question, namely, the synthesis of added substances, even glucose as well as fermentation products; it is now a very large field, which cannot be overlooked or passed over in Pasteur effect considerations, colloquial or otherwise.

The view presented here may well be a matter of some opinion; but it is an interpretation frankly forerunning Meyerhof and Warburg, though so far as possible covering them. We must take into consideration what happened in Pasteur's day, as well outlined in Pasteur's only book "Studies on Fermentation"—a book of remarkable insight as well as experimental command.

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